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CYTOLOGICAL, PHYSIOLOGICAL AND MOLECULAR CHARACTERISATION OF SPARKLING WINE YEASTS

José Miguel Sebastião Fernandes Batista

MESTRADO EM MICROBIOLOGIA APLICADA

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Dissertação orientada pelo Prof. Doutor Rogério Tenreiro

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Esta dissertação de mestrado integra-se no Projecto LEVEc*hamp* (SI I&DT nº 8383), que tem como objectivo o desenvolvimento de soluções integradas para introduzir melhorias no processo de produção da empresa Proenol e consolidar a sua imagem a nível internacional.

O projecto é financiado pelo QREN, no âmbito do Programa Operacional Factores de Competitividade e Fundo Europeu de Desenvolvimento Regional.

ACKNOWLEDGMENTS

First of all, I want to thank Proenol - Indústria Biotecnológica, Lda. for the opportunity to carry out this thesis. A special thanks to Eng. Filipe Centeno for being always available and providing the necessary support during the whole course of this thesis project.

Em primeiro lugar, quero agradecer à Proenol - Indústria Biotecnológica, Lda. pela oportunidade de realizar esta tese. Em especial, agradeço ao Eng. Filipe Centeno pela contínua disponibilidade e todo o apoio no decurso do projecto desta tese.

Agradeço também:

Ao Chefe, *aka* O Grande Chefe, *aka* O Senhor do Gabinete (no offense!), Prof. Dr. Rogério Tenreiro, pela oportunidade que me proporcionou, o conhecimento que me transmitiu, as discussões de carácter científico, as conversas menos científicas, o convívio, a orientação, o apoio, as ideias, a tolerância, a confiança que depositou em mim. Muchíssimas gracias.

À Chefe, Profª. Drª. Ana Tenreiro, por toda a ajuda, absolutamente indispensável. Estou-lhe imensamente grato.

Ao Daniel, à Profª. Drª. Ana Reis e a todos os que frequente ou ocasionalmente me socorreram nos infindáveis dias de amostragem. O meu sincero agradecimento.

Ao Daniel (já tens duas referências directas; do you feel lucky, punk?), à Inês, à Daniela, à Sandra, ao Ricardo e quiçá a outros (maybe you, reader?) – elementos nobres do laboratório. Pela camaradagem, boa disposição, conflitos de interesses (these benches, I own them all), ajuda, partilha, risos, ciência, conversas da treta... Gostei, gostei bastante.

A todos os que disponibilizaram bolinhos, bolachas, chocolates, etc. etc. para consumo do povo, sem os quais eu teria seguramente esgotado as minhas reservas de energia em diversas ocasiões. May your God bless you.

A todos os que de algum modo me apoiaram e confortaram nos momentos críticos.

À minha família, pelo que acabei de referir, e porque a *família* tem toda a importância deste mundo.

E à Catarina, por tudo. Let the sky be no limit to our dreams.

Peace.

ABSTRACT

Sparkling wines represent a distinctive wine type and are highly valued worldwide. They are produced through a second alcoholic fermentation of a base wine, performed by selected strains of *Saccharomyces cerevisiae*, inside a closed vessel, which in the case of the traditional method is the bottle ultimately delivered to the consumer. Once in the base wine and during the second fermentation, yeasts are forced to endure very stressful conditions, such as high ethanol contents (8-12%) and temperatures usually below 16ºC. Very low temperatures (9-10ºC), not uncommon in large underground cellars, are particularly problematic, being an important cause of sluggish or stuck fermentations even when yeasts are properly acclimatized before inoculation. The work herein described had the objective of studying sparkling wine yeasts' physiological changes and adaptation behaviour under very low temperature in-bottle second fermentations. For the purpose, two yeast strains and two base wines were used in small scale in-bottle second fermentations at 10ºC, mimicking actual traditional method production conditions. Each strain was separately inoculated into each of both base wines, and in all four experimental conditions a multiparametric characterisation was conducted throughout the fermentation process. This characterisation, broad and integrative, focused on a number of cytological, physiological and molecular properties of yeasts, intending to thoroughly assess the evolution and heterogeneity of their vitality and adaptation state. Parameters evaluated were: viability, fermentation kinetics, total proteins, trehalose, glycogen, neutral lipids, and expression levels of selected genes (*HSP12*, *GPD1*, *GSH1* and *TRX2*). Both strains showed similar fermentative performances, and no specific cell properties and/or adaptation responses could be pointed as particularly relevant in favouring or hindering a second fermentation at very low temperatures. To better clarify this issue, further analogous studies should be performed with additional strains known or expected to exhibit higher and/or lower fermentative performances at the tested conditions.

Keywords: Sparkling wines; second fermentation; *Saccharomyces cerevisiae*; traditional method; very low temperatures; multiparametric characterisation.

RESUMO

Os vinhos efervescentes, cujo exemplo clássico é o *Champagne*, são bastante apreciados em todo o mundo. A sua produção baseia-se numa segunda fermentação alcoólica de um vinho base (obtido a partir de uma primeira fermentação alcoólica em mosto) em recipiente fechado, invariavelmente realizada por estirpes seleccionadas da levedura *Saccharomyces cerevisiae*. Segundo o método clássico (*méthode champenoise*/*traditionnelle*), usado na produção de *Champagne* e outros vinhos efervescentes de elevada qualidade, a segunda fermentação ocorre no interior da garrafa que será disponibilizada ao consumidor final. O vinho base, suplementado com açúcar fermentável e nutrientes adicionais em quantidades apropriadas, deve inocular-se com leveduras previamente aclimatizadas. A aclimatização ("pé-de-cuba") permite que as leveduras se adaptem e melhor tolerem as condições ambientais hostis que existem ou surgem no vinho base durante a segunda fermentação, entre as quais: teores iniciais de etanol de 8-11% v/v, que aumentam 1-1.5% durante o processo; temperaturas habitualmente inferiores a 16ºC; pressões de CO_2 elevadas, atingindo 5-6 bar no final da fermentação. As leveduras para segunda fermentação encontram-se disponíveis comercialmente como culturas líquidas ou formas secas activas, estas últimas livres ou encapsuladas. As formas encapsuladas foram recentemente introduzidas no mercado e apresentam inúmeras vantagens, como a inoculação directa sem aclimatização prévia. Para a segunda fermentação, as garrafas são preferencialmente mantidas a 10-15ºC. A cinética fermentativa depende de vários factores, entre os quais a estirpe utilizada, o seu estado fisiológico aquando da inoculação, a quantidade de inóculo e as condições físico-químicas no vinho base. Uma vez concluída a fermentação, que a 11-12ºC pode demorar um mês ou mais, o vinho é envelhecido na garrafa sobre o depósito de leveduras, apenas removido no final do processo.

Certas condições de segunda fermentação são particularmente adversas para as leveduras, como é o caso das temperaturas muito baixas (9-10ºC), não raramente encontradas em extensas caves subterrâneas. A estas temperaturas, mesmo uma aclimatização adequada nem sempre permite prevenir fermentações lentas ou paradas. Infelizmente, aumentar a temperatura nas referidas caves é economicamente inviável. Torna-se assim relevante analisar e compreender as alterações fisiológicas que ocorrem nas leveduras durante o processo de segunda fermentação a muito baixas temperaturas. O trabalho descrito nesta tese teve como objectivo estudar as referidas alterações. Para tal, duas estirpes de vinificação de vinhos efervescentes em forma encapsulada, denominadas 1 e 2, e dois vinhos base, designados 1 e 2, foram utilizados para segunda fermentação em garrafa (segundo o método clássico) em pequena escala, a 10ºC, em condições que mimetizaram a produção industrial. Cada estirpe foi separadamente inoculada em cada um de ambos os vinhos base, e em todas as quatro situações experimentais se realizou uma caracterização multiparamétrica das leveduras durante o processo fermentativo, de acordo com os seguintes tempos amostrais: imediatamente antes da inoculação (dia 0), e depois aos dias 1 (24 h), 2 (48 h), 12, 19, 26, 58 e 85 pós-inoculação. A referida caracterização, extensa e integrativa, focou-se em propriedades citológicas, fisiológicas e moleculares das leveduras, com o intuito de avaliar a evolução e heterogeneidade da sua vitalidade e estado adaptativo. Analisaram-se os seguintes parâmetros: viabilidade; cinética fermentativa; proteínas totais; trealose; glicogénio; lípidos neutros;

e níveis de expressão relativa de genes de resposta ao *stress* (*HSP12*, *GPD1*, *GSH1* e *TRX2*). A viabilidade foi estimada de dois modos: pelo método do azul de metileno (MB) e através de citometria de fluxo (FC) com um kit comercial de determinação de viabilidade (baseado em dois corantes fluorescentes). A cinética fermentativa determinou-se a partir da quantificação da glucose (com kit comercial) e glucose/frutose (realizada pela empresa Proenol, que colaborou no estudo) no vinho. As proteínas totais dosearam-se pelo método do biureto após extracção celular. A trealose, carbohidrato de reserva e importante factor de protecção contra diversos *stresses*, analisou-se de duas formas: *a)* foi extraída das células e quantificada indirectamente (quantificação absoluta, AQ), por hidrólise e quantificação da glucose resultante (com kit comercial); *b)* foi avaliada por FC, com utilização de um reagente fluorescente comercial. Os valores médios de fluorescência (FC) foram utilizados para comparação com os valores AQ. O coeficiente de variação robusto (RCV) das distribuições de fluorescência e as formas dos respectivos histogramas foram utilizados na análise da heterogeneidade populacional em teores de trealose. O glicogénio, principal carbohidrato de reserva, analisou-se de modo semelhante à trealose, com: *a)* quantificação da glucose obtida após extracção celular e hidrólise (AQ); *b)* estudo em FC, com utilização de acriflavina (corante fluorescente). Os lípidos neutros, utilizados na biogénese membranar e como reserva energética, foram analisados por FC, com um reagente fluorescente comercial. Os níveis de expressão relativa de *HSP12, GPD1, GSH1* e *TRX2* foram determinados com recurso à técnica de RT-PCR quantitativo em tempo real, utilizando o método $\Delta\Delta C_T$ com correcção para as eficiências de amplificação. *18S* (*RDN18*) foi aplicado na normalização dos valores de expressão. As expressões relativas foram calculadas em relação ao dia 0 (referência), separadamente para cada estirpe. *HSP12* é um gene de resposta geral ao *stress*; *GSH1* e *TRX2* são considerados marcadores de *stress* oxidativo; *GPD1* é expresso em resposta a *stress* hiperosmótico. A análise estatística dos resultados baseou-se em testes *t* de Student, análises de variância (ANOVA) com dois factores e análise de componentes principais (PCA).

As duas estirpes em estudo exibiram um desempenho fermentativo muito semelhante, independentemente do vinho. As fermentações realizaram-se mais rapidamente no vinho 2 (constatou-se o seu término dia 58) do que no vinho 1 (concluídas ao dia 85). Relativamente à viabilidade celular, observou-se uma excelente correlação ($r\approx0.97$; $n=54$) entre os valores obtidos por MB e FC. Dia 26, quando bastante açúcar já havia sido fermentado, ambas as estirpes possuíam ainda uma elevada viabilidade (em ambos os vinhos), que apesar de ter diminuído continuamente ao longo do processo fermentativo não o comprometeu. No que respeita às proteínas totais, constatou-se o seu aumento desde os dias 1 ou 2 até aos dias 19 ou 26, reflexo de um metabolismo activo. A sua quantidade diminuiu depois até ao dia 85, em todas as condições experimentais. Na análise da trealose, verificou-se uma ausência de correlação ($r\approx0.20$; $n=62$) entre os valores de AQ e FC. De acordo com os resultados AQ, houve um aumento substancial dos teores de trealose nas primeiras 24 h, seguido de um decréscimo até às 48 h, em todos os casos. Esta ocorrência poderá traduzir uma resposta inicial ao *stress* causado pelo etanol. Níveis máximos de trealose (AQ) foram observados dias 19 e 26. Crê-se que a sua acumulação terá sido induzida pelas contínuas condições de *stress* no vinho e/ou pela necessidade de reservas de carbono/energia. Os conteúdos de trealose diminuíram depois até dia 85, particularmente em

fermentações no vinho 2, revelando o seu consumo face à depleção progressiva de açúcar no meio. No estudo da trealose por FC constaram-se alterações nos valores dos RCVs ao longo do processo fermentativo em todos os casos, sugestivas de uma heterogeneidade populacional dinâmica. Adicionalmente, os histogramas das distribuições de fluorescência apresentaram-se globalmente diferentes entre estirpes. No caso do glicogénio, registou-se uma correlação fraca entre AQ e FC ($r \approx 0.52$; $n=62$). Teores máximos (AQ) observaram-se geralmente dia 58. Entre os dias 58 e 85, o polissacárido foi consumido em quantidades substanciais, claramente em resposta à escassez de açúcar nos vinhos. Durante o processo fermentativo, além de alterações nos RCVs (análise FC), observaram-se diferenças claras entre os histogramas de ambas as estirpes e de cada estirpe individualmente. Relativamente aos lípidos neutros, detectou-se a sua diminuição nas primeiras 48h na estirpe 1. Esta diminuição, possivelmente causada pela necessidade de reparação membranar, não foi todavia observada na estirpe 2. Nova diminuição, comum a ambas as estirpes, ocorreu entre os dias 12 e 19, provavelmente reflexo de uma reestruturação membranar (adaptativa). A acumulação verificada no final do processo, entre os dias 58 e 85, sugere que os lípidos neutros não foram utilizados como reserva energética neste período. Uma heterogeneidade populacional dinâmica foi novamente constatada, observando-se RCVs variáveis e diferenças nos histogramas ao longo do processo fermentativo (em cada estirpe e entre ambas). Quanto aos níveis de expressão relativa de *HSP12*, *GPD1*, *GSH1* e *TRX2*, verificou-se uma redução considerável nos mesmos durante as primeiras 24 h, em todos os casos. Esta redução sugere que as leveduras já se encontravam bem adaptadas às condições de fermentação. Contudo, observou-se um aumento importante na expressão de todos os genes às 48 h (especialmente *GSH1* e *GPD1*), de novo em todas as condições. Este aumento poderá ter sido induzido pela exposição continuada a factores de *stress* nos vinhos. Novos decréscimos gerais na expressão de todos os genes foram observados entre os dias 2 e 12, e também entre os dias 26 e 85, tendo os mais baixos níveis de expressão sido detectados no último tempo amostral. O último decréscimo poderá ter ocorrido por diferentes motivos, como uma diminuição na estabilidade do mRNA e/ou um comprometimento do processo de transcrição. Na PCA, a variação e separação entre amostras revelou ser bastante dependente do tempo, i.e. da progressão do processo fermentativo. No plano dos componentes principais 1 e 2 (PC1/PC2, explicativos de 83.1% da variância global), a separação entre amostras foi suportada por: *a)* mudanças nos teores de trealose (AQ), reflectidos em PC2; *b)* diminuições na viabilidade e nos açúcares fermentáveis no vinho, evidenciados em PC1. Foi possível verificar que a estirpe 2 teve um comportamento global mais semelhante em ambos os vinhos do que a estirpe 1.

Uma vez que as duas estirpes apresentaram um desempenho fermentativo análogo, torna-se difícil identificar propriedades celulares ou respostas adaptativas específicas (de entre as estudadas) que possam ser particularmente relevantes no decurso da segunda fermentação a muito baixas temperaturas. Deverão realizar-se novos estudos para melhor clarificar esta questão, com recurso a estirpes com capacidades fermentativas distintas nas condições testadas. Por outro lado, parâmetros adicionais (ex.: ácidos gordos e fase do ciclo celular) deverão ser considerados em análises futuras, pela informação complementar que poderão providenciar.

Palavras-chave: Vinhos efervescentes; segunda fermentação; *Saccharomyces cerevisiae*; método clássico; temperaturas muito baixas; caracterização multiparamétrica.

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INTRODUCTION

1. Sparkling wines

Highly valued worldwide, sparkling wines represent a very distinctive wine type, the classic example of which is Champagne, commonly regarded the most prestigious wine in its category. By definition, sparkling wines are produced through a second alcoholic fermentation of a still wine (called base wine) inside a closed vessel (Ribéreau-Gayon *et al.*, 2006; Martínez-Rodríguez and Pueyo, 2009). This fermentation is invariably carried out by selected strains of *Saccharomyces cerevisiae*, a species with huge economic importance in the food and beverage industries (Borneman *et al.*, 2007). The characteristic effervescence of sparkling wines is due to the presence of high levels of carbon dioxide (CO_2), which arise during fermentation and are kept in the final product. Although wines made effervescent by artificial carbonation (through injection and saturation with CO_2) exist, they are regarded as unworthy of serious attention and fall outside the definition given above.

1.1. First fermentation

A first alcoholic fermentation is required for the production of base wine from grape must. Only afterwards, and in a separate process, will a second fermentation process allow the elaboration of sparkling wine (from previously obtained base wine).

Grape must to be used in a first fermentation should be adequately clarified after its extraction, typically by being left to settle down naturally. High levels of unstable proteins may be present, and these are responsible for undesired turbidity; this problem may be overcome by adding moderate quantities of tannins and/or bentonite, which promote protein flocculation (Ribéreau-Gayon *et al.*, 2006). Must may be subjected to further treatments or adjustments, depending on its properties and the intended final quality (Jackson, 2000; Zoecklein, 2002).

Typically, the first fermentation is carried out by selected *S. cerevisiae* strains, and usual procedures in white wine vinification are followed (Jackson, 2000; Martínez-Rodríguez and Pueyo, 2009). New base wines are clarified, filtered and commonly subjected to other treatments (Ribéreau-Gayon *et al.*, 2006). Producers wish to obtain base wines with certain characteristics, such as specific organoleptic properties (*e.g.* pale colour[1] and fruity aromas), low residual sugar content[2] (below 2 g/l), moderate alcohol content (no higher than 10-11% v/v) and low volatile acidity (Ribéreau-Gayon *et al.*, 2006; Martínez-Rodríguez and Pueyo, 2009). Because individual base wines seldom possess all the features producers desire, their blending is a very common practice for sparkling wine production; base wines from different vineyards, grape varieties and even vintages are often combined in specific ways. In addition to improving the overall quality of the sparkling wine, blending helps minimize yearly variations in supply and quality.

[1] Most sparkling wines are white, being produced from white base wines.

[2] For the production of some sparkling wines, especially by the Bulk/Charmat method, a higher sugar content may be wished (Jackson, 2000); in these cases, primary fermentations are stopped prematurely.

1.2. Second fermentation

Different sparkling wine production methods exist, the differences between which are largely associated with the second fermentation step and subsequent procedures. A second fermentation can take place in bottle, according to either the *méthode champenoise* / *méthode traditionnelle* (commonly referred to as traditional method, described later) or the Transfer method, as well as in large containers, by the Bulk/Charmat method. Second fermentation vessels (bottle or container) must remain hermetically sealed during the process.

1.2.1. Sparkling wine yeasts (second fermentation yeasts)

Desired characteristics

Regardless of the sparkling wine production method, the second fermentation is typically performed by selected strains of *S. cerevisiae*, which are inoculated in the base wine (usually blended and enriched with fermentable sugar and additional nutrients). These yeasts must be able to withstand the very unfavourable conditions that are present or develop in the base wine during the second fermentation: initial ethanol contents between 8% and 11% v/v, which raise by 1-1.5% throughout the process; temperatures usually lower than 16ºC; pH below 3.3; high CO_2 pressures, reaching 5-6 bar at the end of fermentation; and free sulphur dioxide (SO_2) contents up to 25 mg/l (Jackson, 2000; Zoecklein, 2002). Importantly, besides being capable of rapidly initiating and successfully and consistently finishing the second fermentation within the expected time period, the selected strains must contribute in a positive way to the overall organoleptic properties of the final product. In this regard, yeasts should not produce detectable quantities of hydrogen sulphide (H_2S) or other unwanted volatile sulphur-containing compounds, which are a major cause of off-flavours and undesirable odours. Low tendency to produce acetaldehyde, ethyl acetate, acetic acid and SO_2 is also wished (Jackson, 2000). On the other hand, selected yeasts should have a satisfying effect on carbonation (Zoecklein, 2002). If traditional method is used, yeasts should also flocculate well, which facilitates their removal from the bottle after fermentation (and aging), done by riddling and disgorging (more on this later). Due to the demanding and sophisticated modern wine markets, yeast strains possessing any optimized, improved or novel oenological properties are frequently sought for.

Available preparations

Wine yeasts used for second fermentations are commercially available in liquid cultures and active dry forms (Krieger-Weber, 2009). Regarding the latter, two different options exist: *free* active dry yeasts (ADY) and encapsulated (*immobilized*) active dry yeasts.

Liquid yeast cultures

Liquid yeast cultures have a very limited market, mainly because of their short shelf-life; yeast viability, though very high in fresh cultures of this kind, rapidly declines if further propagation is not performed. Suppliers of liquid cultures include some wineries and wine laboratories or institutes (Krieger-Weber, 2009).

Before being added to a base wine, these cultures must be properly acclimatized (and propagated as needed). The procedure allows yeasts to gradually adapt to and better tolerate the unfavourable conditions in the base wine, especially the high ethanol contents and low incubation temperatures. If acclimatization is not performed, most yeast cells become severely damaged and/or die once in the base wine, and there is an extended lag/adaptation phase (Jackson, 2000). Ultimately, this may cause fermentation to slow down (sluggish fermentation) or effectively stop (stuck fermentation), which are undesirable results. Slightly different acclimatization protocols have been described (Jackson, 2000; Zoecklein, 2002; Krieger-Weber, 2009). Generally, the yeast culture is inoculated in a diluted base wine supplemented with sugar and additional nutrients, and initially incubated at 20-25ºC. Once this new culture is actively growing, it may be further propagated in less diluted base wine mixtures and finally in undiluted base wine. The full procedure may take several days to finish. Throughout the acclimatization process, culture temperature should be progressively reduced toward the temperature at which the second fermentation will occur (Jackson, 2000; Zoecklein, 2002). It is crucial to avoid temperature differences of more than 5ºC between acclimatized yeast cultures (starter culture) and the final base wine to which it will be transferred (Krieger-Weber, 2009). Culture aeration (by stirring) during acclimatization is also very important, as it stimulates yeast cell growth and assures adequate production of unsaturated fatty acids and sterols, necessary for proper membrane structure and function and cell division (Jackson, 2000). Importantly, synthesis and incorporation of ergosterol and unsaturated fatty acids in cell membranes is associated with an increased ethanol tolerance (Ding *et al*., 2009).

Free *active dry yeasts (ADY)*

Free active dry yeasts (ADY) first appeared in the winemaking market more than 30 years ago (Fugelsang and Edwards, 2007) and their use increased considerably within the last two decades (Ribéreau-Gayon *et al*., 2006). Nowadays, major wine yeast commercial suppliers mostly sell ADY, making available more than 200 strains (Soubeyrand *et al*., 2006). Moreover, ADY have become the first choice of many winemakers, including sparkling wine producers. The use of ADY for second fermentations has been considered easier (Zoecklein, 2002) and more suitable (Ribéreau-Gayon *et al*., 2006) than that of traditional yeast culture preparations (liquid cultures, for instance).

A very significant advantage of ADY over fresh cultures is their increased shelf-life. ADY can be stored for extended periods of time, often several months, without considerable loss of viability and fermentation capability (Fugelsang and Edwards, 2007). Nonetheless, this extended shelf-life greatly depends on storage conditions. Commercially available ADY packages must be kept unopened until use, so as to avoid ADY exposure to oxygen and moisture, and at constant low temperature (4ºC) (Redón *et al*., 2008). Due to their low moisture content (normally 8% or less), ADY should be properly rehydrated before use. Rehydration is a critical step: ADY viability, physiological state and fermentation performance are significantly influenced by the rehydration conditions used (Soubeyrand *et al*., 2006; Krieger-Weber, 2009). Manufacturer's instructions vary, but most recommend rehydrating ADY in water or in a mixture of water and grape juice/must (1:1) at 37-40ºC, during 15 to 20 minutes (Fugelsang and Edwards, 2007). Rehydrating ADY in the presence of inactive yeast preparations naturally rich in sterols (and other nutrients) has a very

positive impact on their viability and vitality; sterols seem to be particularly important for this effect, probably by helping to restore membrane's structure and function, affected by drying and rehydration (Krieger-Weber, 2009). Once rehydrated, ADY should also be subjected to a suitable acclimatization before inoculation in base wine. If these yeasts are not in an adequate physiological state at the time they are put in the base wine, they may not be able to cope with the known stressful conditions, and fermentation will be compromised. Acclimatization procedures vary (Carvalheira, 2008; Krieger-Weber, 2009); nevertheless, they are equivalent to the ones for non-ADY cultures, already referred. Krieger-Weber (2009) described one widely used protocol for ADY (Krieger-Weber, 2009). Again, particular attention should be given to acclimatization temperatures and aeration.

Encapsulated yeasts

Encapsulated yeasts represent a significant breakthrough in wine industry. They were developed and recently introduced in the market by Proenol, a Portuguese company. Different encapsulated yeast products are commercially available, each with a unique winemaking application (Proenol, 2010a). One of them, brand-named ProElif, is especially intended for, and extremely helpful in, the production of sparkling wine by the traditional method.

Encapsulated yeasts consist of yeast cells physically entrapped (therefore *immobilized*) within a double-layered calcium alginate capsule, presented as small dehydrated beads with an average 2 mm diameter (Proenol, 2010a; Proenol, 2010c). In the particular case of ProElif, the beads can be directly inoculated in a base wine, eliminating the need to previously rehydrate, propagate and acclimatize yeasts (Proenol, 2010c). This is extremely practical, and a remarkable advantage over ADY. The alginate capsule, although solid, is in fact a porous matrix; this allows yeast cells to be in permanent contact with the base wine, and substrates and metabolites to easily diffuse throughout the matrix. Importantly, yeast cells are effectively held inside the capsule (Proenol, 2010c). The average number of viable cells per bead weight is known, and this helps ensure control over the number of viable cells per bottle. Another major advantage of encapsulated yeasts over ADY (or other starter cultures) is that they let producers skip the conventional riddling step: inverting the bottle is sufficient for the beads to quickly fall in the bottleneck to be removed. Suppressing riddling considerably reduces sparkling wine production costs and labour. Also, as most riddling equipment becomes unnecessary, more space is available in the cellar. Finally, and most importantly, sparkling wines produced with encapsulated yeasts show similar organoleptic properties to the ones produced with free yeasts (Proenol, 2010b; Proenol, 2010c).

As said above, commercial distribution of encapsulated yeasts for in-bottle sparkling wine production is fairly recent. However, the idea of encapsulating yeasts and using them for this enological process is by no means new (Fumi *et al*., 1987), and the procedure was particularly envisaged to skip riddling. For several years, the potential of this experimental technology was studied by different research groups (Fumi *et al.,* 1988; Gòdia *et al*.,1991; Yokotsuka *et al*., 1997). An interesting (but somewhat dated) review on the subject, which also addressed other wine yeast immobilization technologies and thoroughly considered the physiology and activity of immobilized

yeast cells, was published by Martynenko and Gracheva (2003), still before encapsulated yeasts started to be commercialized.

1.2.2. Traditional method (*méthode champenoise* / *traditionnelle*)

In the case of Champagne wine, the second fermentation takes place in the bottle that will ultimately be delivered to the consumer, according to a method known as *méthode champenoise*. Champagne production follows the *Appellation d'Origine Contrôlée* (Controlled Designation of Origin) regulations. Using the expression *méthode champenoise* for sparkling wines produced, by the same method, outside the french region delimited by the *Apellation* is now prohibited and has been commonly replaced by *méthode traditionnelle* (Ribéreau-Gayon *et al*., 2006). The best quality and most prestigious sparkling wines are made by the *méthode champenoise* / *traditionnelle*, also known as traditional method. Its different steps are described below.

Preparing and bottling the cuvée *(base wine)*

The base wine used in the production of Champagne is typically blended and referred to as *cuvée*. After blending, the *cuvée* is cold-stabilized to prevent tartrate precipitation, which can influence taste and gas release in the final product (Zoecklein, 2002*)*. It may also be filtered and/or fined (Ribéreau-Gayon *et al*., 2006). Before or during the bottling process, a defined quantity of a concentrated sucrose syrup called *tirage liqueur* is added to the *cuvée*, providing the necessary fermentable sugar[3] for the second fermentation to originate CO_2 levels required for a final pressure of 5-6 bars inside each bottle[4] (Jackson, 2000; Ribéreau-Gayon *et al*., 2006). If the *cuvée* already contains fermentable sugar, this must be taken into account before *tirage liqueur* addition. Besides sucrose, the *tirage liqueur* may have additional nutrients. Many producers also add a source of assimilable nitrogen[5] to the *cuvée*, commonly 100 mg/l or more of diammonium hydrogen phosphate, known as DAP (Jackson, 2000; Zoecklein, 2002; Carvalheira, 2008*)*. Assimilable nitrogen is essential for the growth and fermentative activity of yeasts; its deficiency may limit the rate of fermentation and lead to a sluggish or stuck process. Considering only yeast nutritional requirements, addition of assimilable nitrogen to the *cuvée* is unnecessary if its initial concentration is above 15 mg/l (100 mg/l of DAP provide about 27 mg/l of assimilable nitrogen). Supplementation may, however, help to diminish H_2S production (Jackson, 2000). Trace amounts of copper salts (≤0.5 mg/l) are sometimes added to help neutralize H_2S. Some producers also incorporate one or more substances to facilitate yeast flocculation and removal (*e.g.* bentonite) when bottles are disgorged.

To inoculate the *cuvée*, properly acclimatized yeasts should be used. Some producers choose to inoculate ADY immediately after rehydration (Carvalheira, 2008); however, precaution must be taken, as this can create fermentation problems. If encapsulated yeasts are used, they can be

[3] Sucrose ends up being hydrolyzed to glucose and fructose.
[4] A final concentration of about 24 g/l of sucrose in the *cuvée* allows the production of a ~6 bar pressure inside a standard 750 ml bottle, at 10ºC (Ribéreau-Gayon *et al*., 2006).
[5] Assimilable nitrogen is defined as nitrogen in the form of ammonia and α-amino nitrogen of amino acids other than proline (Rossignol *et al*., 2003).

promptly introduced in the *cuvée*. It is important to inoculate an adequate number of cells for an effective and efficient onset and completion of fermentation. Recommendations vary: Ribéreau-Gayon *et al*. (2006) suggest an initial yeast population of 1.5 x 10^6 cells/ml, whereas Jackson (2000) points 3-4 x 10^6 cells/ml as advisable. Encapsulated yeasts should be inoculated in slightly higher numbers (Proenol, 2010c). Importantly, a threshold initial cell number exists below which fermentation becomes slower and may prematurely stop. On the other hand, too high cell numbers may increase the production of H_2S and additional off-odours (Jackson, 2000; Ribéreau-Gayon *et al*., 2006), although they also accelerate the process. If the yeast culture to be inoculated in the *cuvée* is in liquid medium, inoculation volume must be accounted for (generally 2-5% of the final volume).

Once the *cuvée* has been mixed with the *tirage liqueur*, inoculated and bottled, bottles are closed with crown caps made airtight by plastic seals (Ribéreau-Gayon *et al*., 2006).

Second fermentation and aging

For the second fermentation, bottles should be kept at a low, stable temperature, preferably 10-15ºC (Jackson, 2000). Moreover, light exposure is to be avoided. Several bottle storage systems exist (Zoecklein, 2002); importantly, bottles should be placed in a horizontal position, as this provides the maximum interface area between yeast cells and the *cuvée*. The second fermentation rate highly depends on several factors, namely: yeast strain; yeast's initial cell number, viability and physiological state; temperature; CO_2 pressure; and *cuvée* chemistry (ethanol content, SO_2 levels, pH and existent nutrients) (Jackson, 2000; Zoecklein, 2002*;* Ribéreau-Gayon *et al*., 2006). At a constant temperature of 11-12ºC, a second fermentation may take approximately one month, but sometimes longer, to finish (Ribéreau-Gayon *et al*., 2006). These slow, smooth, low temperature fermentations are an important quality factor in producing fine sparkling wines in the Champagne region.

Once the second fermentation is complete (i.e. all sugar consumed), the wine is left to spend a long time aging inside the bottle, still in contact with yeast cells (Ribéreau-Gayon *et al*., 2006). At this point, the yeast sediment is frequently referred to as "lees", and the aging process is known as "aging on lees". When fermentation finishes, and throughout the aging process, the number of viable yeast cells rapidly declines (Jackson, 2000). Furthermore, yeasts undergo autolysis, a process characterised by the hydrolysis of cellular biopolymers under the action of hydrolytic enzymes, which are then activated. This results in a gradual release of several cellular breakdown components into wine, such as peptides and amino acids, fatty acids, nucleotides, glucans and mannoproteins (Pérez-Serradilla and de Castro, 2008; Pozo-Bayón *et al*., 2009). Autolysis is of great importance in lees composition and on their overall influence on wine properties. Lees have a seemingly relevant antioxidant capacity, to which yeast cell wall components (thiols, mannans and others) and adsorbed wine polyphenols contribute (Gallardo-Chacón *et al*., 2010). In fact, it is known that as long as sparkling wines remain in contact with lees under anaerobic conditions, they are perfectly preserved (Ribéreau-Gayon *et al*., 2006). Aging on lees may have a significant impact on the final wine quality; generally, it is associated with an overall improvement in wine organoleptic properties (Ribéreau-Gayon *et al*., 2006; Pérez-Serradilla and de Castro, 2008). Aging normally

lasts around 9 months, but may continue for several years, depending on the wine characteristics producers desire (Jackson, 2000).

Riddling, disgorging and corking

Following aging, lees are gathered on the inside of the bottle cap. This is done by riddling the bottles. The process consists of slightly turning the bottles and gradually bringing them to a vertical position. Riddling is labour-intensive and expensive if manually performed. Also, it requires plenty of space for riddling racks (Ribéreau-Gayon *et al*., 2006). Manual riddling is rapidly disappearing, as most wineries choose automated mechanized riddling equipment. While manual riddling may take 1 month to finish, automated riddling is much faster, ending in about 1 week. As mentioned before, encapsulated yeasts are a very practical solution, allowing to simply skip riddling. Once lees are settled on the cap, bottles are disgorged. For disgorging, bottle necks are first immersed in a low temperature salt solution, which freezes about 2 cm of wine above the cap, trapping the lees sediment (Ribéreau-Gayon *et al*., 2006). A disgorging machine then rapidly removes the cap and the frozen lees, and other devices adjust wine volume (Jackson, 2000). Most sparkling wines have a *dosage liqueur* added at this step, which typically consists of a concentrated sucrose solution dissolved in high-quality aged wine. Lastly, the bottles are sealed with special corks, only to be opened by the final consumer (Jackson, 2000).

2. Monitoring the second fermentation

2.1. A problem-solving multiparametric analysis?

As previously mentioned, sparkling wine yeasts are forced to endure very stressful conditions once in the base wine and throughout the whole second fermentation. Therefore, if their physiological state is not optimal at the time they are inoculated, they may not be able to successfully withstand those conditions. In practice, this will cause the fermentation to slow down, abruptly stop, or even not start at all. Fortunately for producers (and consumers), the problem may be minimized or prevented in many cases, with an adequate rehydration (in the case of ADY) and/or acclimatization of yeasts, which allows them to adapt to and better cope with the hostile base wine environments. Although many situations may in fact be solved this way, others exist that are particularly problematic. Such is the case of second fermentations at very low temperatures, as low as 9º-10ºC (Zoecklein, 2002), not uncommon in large underground cellars, *e.g.* in Champagne region. These temperatures are especially stressful for yeasts, and even an adequate acclimatization cannot always prevent a sluggish or stuck fermentation. Increasing the temperature inside the cellars is not a viable solution, due to the associated high costs with equipment and energy.

Analysing and comprehending yeasts' physiological changes under very low temperature second fermentations therefore becomes important. To clearly and thoroughly understand the effects of these harsh second fermentation conditions on wine yeasts, an extensive and multiparametric analysis should be developed and performed, aimed at studying the evolution and population heterogeneity of yeasts' vitality and adaptation state under those conditions, i.e. during

second fermentations at very low temperature. An approach of this kind must focus on several different cellular parameters, properly selected for their useful informative value and representing cytological, physiological and molecular cell properties. Importantly, results should be considered as a whole, in an integrative way, and an appropriate multivariate analysis should ultimately be performed for their interpretation (Devantier *et al*., 2005). A study of this kind, or a simpler and more practical version of it (*e.g.* less parameters analysed), could be further used to monitor and control any second fermentation, under problematic conditions or not, being particularly useful to follow the physiological state and performance of yeasts in different situations. The procedure could perhaps even be applied, with the needed adjustments, to other yeast-based industrial fermentations.

Information provided by this multiparametric approach could be useful for industrial wine yeast producers (producers of active dry yeast forms). They would probably be in better position to re-evaluate their standard production methods and try to optimize them, in order to produce sparkling wine yeasts even more resilient, capable of performing second fermentations under very difficult conditions. A hypothetical case would be to modify biomass propagation conditions in order to stimulate cellular synthesis of particular stress protectants (such as trehalose, discussed below), determined to be helpful for yeasts survival and initial adaptation in especially difficult second fermentations.

As said above, the multiparametric study should focus on cytological, physiological and molecular properties of yeasts. Regarding a cytological and physiological characterisation, it is important to assess yeast cell viability and quantify cell molecules which may provide relevant information on yeasts' vitality and adaptation state, such as trehalose, glycogen, total proteins, fatty acids and glutathione. Analysing the distribution of at least some of these molecules at a population level could also be relevant, as it would reveal the physiological diversity within yeast populations (and therefore their heterogeneity). Yeasts' fermentative performance, though not a physiological property *per se*, depends on their physiological state and is a good indicator of vitality; obviously, it is a key parameter to follow (through fermentation kinetics analysis). With respect to molecular analysis, it implies determining gene expression levels. The expression of stress responsive genes (associated with one or more stress conditions) is particularly relevant to follow, as it allows a better understanding of yeasts' immediate, delayed and/or maintained stress responses and adaptation state. All these cytological, physiological and molecular parameters are further considered below.

2.2. Cytological and physiological characterisation of yeasts

2.2.1. Viability

Yeast cell viability may be assessed by culturing on suitable solid media under proper incubation conditions and counting "colony-forming units" (Ivorra *et al*., 1999; Zuzuarregui and del Olmo, 2004). However, plate culture based methods take at least 24-48 h to yield results, too much time to be of practical use to monitor fermentation processes. Moreover, starvation and other multiple stresses may induce cells to adopt non-culturable states, though they may still be viable and exhibit metabolic activity to various extents. This activity may be relevant for the course of a fermentation,

and is therefore ignored by traditional culture methods, which rely only on the cell reproductive capability under strictly defined conditions (Díaz *et al.*, 2010).

A common alternative to culturing, traditionally used in brewing and winemaking industries (Boyd *et al*., 2003; Fugelsang and Edwards, 2007), is methylene blue (MB) staining. After cell sample staining, MB allows differentiation of viable and non-viable yeast cells under direct microscopic observation, and cell counts may be performed in a Neubauer counting chamber. Cells with compromised membranes are permeable to the dye and stain blue, being considered dead. Live cells, with intact membranes, either exclude or reduce MB, turning it colourless. MB is reduced at the cell surface, though the exact mechanism is unknown. The reduced form is lipophilic, entering live cells by diffusion across the membrane. If oxygen is available, reduced MB can be re-oxidized by the mitochondrial electron transport system, and this will cause reappearance of the blue colour (Bapat *et al*., 2006). For this reason, and because MB rapidly becomes toxic to cells, stained samples should be analysed within few minutes (Fugelsang and Edwards, 2007). Although relatively inexpensive and easy to perform, this method has several drawbacks: operator fatigue; analysis is subjective, with often variable interpretations of weakly stained cells; and relatively low numbers of cells are counted, usually less than 400, which is not very representative of the entire population (gives an error of at least 5% calculated by $100\sqrt{n}/n$ (%), where n is the number of cells counted) (Boyd *et al*., 2003). Results with MB may differ from culture-based ones (Manzano *et al.*, 2006).

Ideally, an improved, rapid, accurate and reliable method should be used to monitor yeast viability during fermentation processes. Flow cytometry (FC) offers all these advantages, and also the possibility of near real-time monitoring. In FC, individual cells or particles in a directed fluid stream pass through a focused laser beam. Their interaction with the beam generates optical signals of variable intensities, associated with light scattering and fluorescence emission (in this latter case, mostly if fluorescent dyes are used), which are ultimately correlated to structural and/or functional cell parameters (Comas-Riu and Rius, 2009; Díaz *et al.*, 2010). Optical signals are detected, recorded and processed by different integrated systems in a flow cytometer, and acquired data may be graphically visualized as a sample (a cell suspension) is being analysed. Data is usually represented in monoparametric histograms (single parameter frequency distributions) and/or dot plots (biparametric), and can be stored for further analysis (Díaz *et al*., 2010). The power of FC lays both in the possibility of assessing a wide range of cell properties at the single cell level, particularly through the use of different fluorescent dyes, and in the ability to get information about the distribution of those properties within cell populations, therefore providing valuable information on their natural heterogeneity. Additionally, some flow cytometers are able to physically separate (sort) cell subsets based on their optical characteristics, permitting further studies to be conducted on them. In FC, assays are automated (once protocols are established), which eliminates subjective analysis. Plus, individual cells are analysed at rates of hundreds, at times thousands per second; thus, a large number of cells can be quickly examined (easily $\geq 10^4$ per sample), which significantly reduces the error (which is 1% or less).

In order to assess yeast viability by FC, specific fluorescent stains (alone or combined) must be used, being added to cell samples prior to FC analysis. Mixing a defined volume of reference

fluorescent particles (beads) of known concentration with cell samples also allows for simultaneous determination of cell concentrations, through determination of cells/beads ratio. Yeast cells, easily identified by their light scattering signals, are considered viable or non-viable according to their fluorescence(s). Different criteria may be used to estimate viability by FC, such as cell membrane integrity, membrane potential and/or cell enzymatic activities, which require different fluorescent dyes to be utilized (Díaz *et al*., 2010). More than one criterion may be considered simultaneously, an approach that provides broader information on the structural and functional properties of cells and on populations' diversity. Importantly, different vitality states can be discriminated this way.

Evaluation of cell membrane integrity, a common procedure to estimate viability by FC, may be done with fluorescent dye exclusion methods. Basically, a fluorescent dye is used that is excluded by cells with intact membranes (considered live). If membrane integrity is lost, the dye enters cells (considered dead) and fluoresces upon binding their nucleic acids, which it specifically stains. Propidium iodide (PI) is the most commonly used dye for this purpose, either alone (Fiala *et al.*, 1999; Attfield *et al*., 2000) or with a second dye (Chaney *et al*., 2006; Farthing *et al*., 2007) that also stains nucleic acids but the fluorescence of which is different (typically green; PI emits red fluorescence). The second dye (*e.g.* Thiazole orange) is membrane permeable and enters all cells, allowing accurate total cell counts. Using two dyes allows better identification of live and dead cells, and may even reveal a third population of "injured" cells, with an apparent partial (perhaps temporary) loss of membrane integrity (Farthing *et al*., 2007). This clearly demonstrates the potential of FC in revealing different cell states and heterogeneities within populations.

It should be emphasised that several different fluorescent dyes are currently available, which allow FC users to analyse many structural and functional cell properties, such as membrane integrity and potential, metabolic activities (*e.g.* enzymatic activities) and contents of a variety of biomolecules. This permits a deep characterisation of yeast cell populations, which is the purpose of the multiparametric study discussed above.

2.2.2. Fermentation kinetics

Wine yeasts' fermentative performance can be determined through the analysis of fermentation kinetics. Different parameters should be considered on this regard: *1)* initial fermentation delay (may or may not occur), associated with yeasts' lag/adaptation phase and in which little consumption of sugars (glucose/fructose) occurs; *2)* total fermentation length, i.e. time needed for the fermentation to be considered complete (all sugars consumed, or approximately so); and *3)* fermentation rate – maximum rate and rate dynamics –, i.e. speed at which sugars are consumed (Jackson, 2000; Llauradó *et al*., 2002). In the production of still wine (from must), fermentation kinetics can be monitored in different ways, by measuring either: the amount of fermentable sugar still present in must/wine; the amount of ethanol formed or carbon dioxide released; or the density of the must/wine, which provides an estimation of the potential ethanol content (sugar consumption and ethanol formation lowers must/wine density) (Ribéreau-Gayon *et al*., 2006). In in-bottle sparkling wine production, the progress of the second fermentation is generally followed by determining the fermentable sugar still present in base wine and/or the pressure inside bottles (some reference bottles are used for this to be done) (Zoecklein, 2002).

2.2.3. Trehalose

Trehalose is a non-reducing disaccharide composed of two α (1,1)-linked glucose molecules (François and Parrou, 2001). It is considered a storage carbohydrate (along with glycogen) and, most notably, an important stress protectant in yeasts. Also, it is associated with the nutrient-induced control of cell cycle progression and the control of glucose sensing, transport and initial stages of metabolism (Pretorius, 2000). Trehalose synthesis occurs in a two-step process, which involves the formation of trehalose-6-phosphate(P) (Tre6P) from UDP-glucose and glucose-6-P, followed by Tre6P dephosphorylation. The initial step is catalyzed by Tre6P synthase (Tps1p) and the second by Tre6P phosphatase (Tps2p), which are catalytic subunits of the trehalose synthase complex (the remaining subunits are Tsl1p and Tps3p, both regulatory). Trehalose hydrolysis is mediated by either a neutral trehalase (Nth1p), cytosolic, or an acid trehalase (Ath1p), vacuolar. Nth2p, highly homologous to Nth1p, is thought of as a third trehalase, though its function has not yet been demonstrated (François and Parrou, 2001; Novo *et al*., 2005).

It is well recognized that the capacity of yeast cells to withstand several harmful environments is correlated with their trehalose content. It is thought that trehalose has the ability to replace water molecules in yeast membranes, protecting them from desiccation by stabilizing and preserving their structure. Some studies revealed that 2-3% of dry weight of intracellular trehalose are sufficient to greatly improve dried yeasts viability (François and Parrou, 2001), and this may result, at least in part, from membrane protection by trehalose. Also, baker's yeasts with elevated trehalose levels (≥10% of dry weight) are fairly resistant to the drying process (after biomass propagation) in terms of retention of leavening capacity (Walker and Dijck, 2006). Trehalose also seems to perform an important role in fighting ethanol stress (Kim *et al.*, 1996), possibly because it can displace ethanol in yeast membranes (ethanol may substitute for water, compromising membrane structure/function), and in doing so, maintaining their integrity and stability (Lucero *et al*., 2000; François and Parrou, 2001). Furthermore, trehalose is able to exclude water from protein surfaces, thus protecting native proteins from denaturation. This property may also be very important, for example, in response to heat stress, a condition under which trehalose accumulates and acts synergistically with Hsp104[6] to confer thermoprotection (Walker and Dijck, 2006). High levels of trehalose may nevertheless suppress aggregation of already denatured proteins in a way that prevents their refolding by molecular chaperones. On this regard, it was proposed that the synergy between trehalose and some chaperones (*e.g.* Hsp104) in stress tolerance is only effective if trehalose accumulation in response to stress is followed by its quick degradation as soon as cells return to normal conditions (François and Parrou, 2001). A long-term low temperature adaptation improves yeast survival at lower or even freezing temperatures, and this is mostly due to the high accumulation of trehalose, glycerol and molecular chaperones such as Hsp104 and Hsp12 (Walker and Dijck, 2006; Aguilera *et al*., 2007). Accordingly, brewer's yeasts with elevated levels of trehalose are able to maintain cell viability in cold storage conditions (at 4ºC in 5% v/v ethanol) for several days. Barotolerance in yeasts may also be linked to trehalose accumulation (Walker and Dijck, 2006).

[6] Heat shock proteins (HSPs), of which Hsp104 is an example, are responsible for stabilizing, preventing aggregation and assisting refolding of denatured proteins, acting as molecular chaperones (Ding *et al*., 2009).

Because of the significant protective role of trehalose, particularly in response to stressful environmental changes, manufacturers of active dried wine yeasts are generally recommended to propagate their yeasts in such a way as to maximize the amount of trehalose cells accumulate (Pretorius, 2000). Trehalose favours yeasts viability during dehydration, which follows biomass propagation (François and Parrou, 2001; Walker and Dijck, 2006). Most probably, trehalose also has important implications for the viability, vitality and physiological activity of active dried wine yeasts upon rehydration/reactivation and thereafter (Pretorius, 2000). It is thus expected that trehalose may have a significant function in sparkling wine yeasts adaptation to the difficult second fermentation conditions.

Recently, Chlup *et al*. (2008) used FC to assess trehalose (and glycogen) contents of brewing yeasts during wort fermentations. Interestingly, they observed bimodal distributions for trehalose. It could be useful to conduct a FC analysis of intracellular trehalose as part of the intended multiparametric study; it would allow determining trehalose contents distribution and evolution over time at the population level. Since inefficient yeast subpopulations (physiologically disturbed and incapable to ferment) may be present in inoculums and/or arise during the second fermentation, having a negative impact on the overall process, physiological heterogeneity needs to be detected and characterised.

2.2.4. Glycogen

Glycogen is the main reserve/storage carbohydrate in yeast cells. It typically accumulates at the end of the exponential phase of growth, while its breakdown follows the depletion of nutrients at the end of fermentation (Pretorius, 2000). Chemically, it is a high molecular mass branched polysaccharide of linear α (1,4)-glucosyl chains with α (1,6)-linkages (François and Parrou, 2001). Glycogen synthesis involves three steps, briefly: *1)* production, from UDP-glucose, of short α (1,4)-glucosyl chains (initiation/nucleation step, catalyzed by "glycogenin" Glg1p/Glg2p); *2)* formation of α (1,4)-glucosidic bonds from UDP-glucose to the non-reducing end of linear α (1,4)-chains (elongation step, catalyzed by glycogen synthases Gsy1p/Gsy2p); *3)* internal ramification of the linear α (1,4)-glucosyl chains with blocks of 6-8 residues, through α (1,6)-linkages (branching step, catalyzed by amylo (1,4)→(1,6)-transglucosidase Glc3p). Glycogen degradation can occur either by amylolysis, catalyzed by α-glucosidases that produce glucose, or by sequential reactions involving phosphorolysis and debranching activities, which produce glucose-1-P and glucose (François and Parrou, 2001).

Glycogen, whose accumulation by yeasts propagated for drying has been linked to enhanced viability and vitality upon rehydration/reactivation, provides a readily mobilizable carbon and energy source. Producers of active dried wine yeasts are thus usually recommended to cultivate their yeasts in such a way as to maximize the amount of glycogen accumulated by cells, as happens for trehalose (Pretorius, 2000). A greater capacity to produce and accumulate glycogen was reported to contribute to an enhanced viability under glucose deprivation conditions (Pérez-Torrado *et al*., 2002), further demonstrating the important role of this carbohydrate in yeasts physiology.

A few different studies have been published in which FC was used to follow glycogen contents of cultured brewing yeasts throughout a certain time period (Hutter, 2002; Bányik *et al*., 2005;

Novak *et al*., 2007; Chlup *et al*. 2008). Hutter (2002) was able to identify two distinct subpopulations differing in glycogen content under certain suboptimal fermentation conditions ("nutrient limitation"). Chlup *et al*. (2008) also observed bimodal distributions. Like trehalose, glycogen should also be analysed by FC, in order to improve the multiparametric study informative power.

2.2.5. Total proteins, neutral lipids, fatty acids and glutathione

Besides trehalose and glycogen, other yeast cell components may provide relevant information on yeasts' vitality and adaptation state, such as total proteins, neutral lipids, fatty acids, and glutathione.

Yeast cells' total protein contents directly reflect their metabolic state, changing through the course of cell cycle and according to environmental conditions. For example, actively growing and stationary phase cells possess quite different protein levels, which are considerably higher in the first (Majara *et al*., 1998).

Yeasts' neutral lipids are mostly steryl esters (STE) and triacylglycerols (TAG), which accumulate in and form the hydrophobic core of the so called lipid particles (also known as lipid granules or droplets). The neutral lipid core is isolated from the cytosolic environment by a phospholipid monolayer possessing a small amount of characteristic proteins embedded. Neutral lipids are stored as an energy reserve and a source of building blocks for membrane lipid biogenesis (sterols and fatty acids), and can be quickly mobilized to fulfil cell's needs (Daum *et al*., 2007). Moreover, the accumulation of lipid particles may be considered a survival mechanism involved in the detoxification of excess sterols, sterol precursors and/or fatty acids, which could cause membrane perturbations (Mannazzu *et al*., 2008). As with trehalose and glycogen, yeasts' neutral lipids analysis by FC has also been described (Mannazzu *et al*., 2008; Gaspar *et al*., 2008).

Cell fatty acid composition seems to be one important factor involved in wine yeasts' survival and adaptation to difficult fermentation conditions. Mannazzu *et al*. (2008) observed that higher ratios of C16 monounsatured and total unsaturated to total fatty acids positively influenced the membrane integrity and viability of wine yeasts during fermentation in must. This may have happened due to higher ethanol tolerance, which correlates with high levels of unsaturated fatty acids in the cell membrane (Ding *et al*., 2009; Krieger-Weber, 2009*)*. Yeast cells' ability to properly modulate their fatty acid composition is therefore relevant under winemaking conditions. Environmental factors such as temperature and culture media composition clearly influence fatty acid metabolism (Beltran *et al*., 2008).

Glutathione is a tripeptide (L-γ-glutamyl-L-cysteinylglycine) that may exist in a reduced (GSH) or oxidized (GSSG) state, though most of it is usually maintained in a reduced form inside yeast cells. It is synthesized from its constituent amino acids via two ATP-dependent steps, the first of which is catalyzed by γ-glutamylcysteine synthetase (Gsh1) and the second by glutathione synthetase (Gsh2) (Grant, 2001). Glutathione performs different important roles in yeast cells (Penninckx, 2002), the most significant of which is perhaps its protective effect against oxidative stress. Yeast strains lacking GSH or with diminished GSH/GSSG ratios are sensitive to this type of stress when it is caused by peroxides or superoxides (reactive oxygen species) and products of cell lipid peroxidation (Grant, 2001). GSH is oxidized to GSSG upon reacting with those and other

molecules, and regenerated in an NADPH-dependent reaction. Garre *et al*. (2010) observed that glutathione may have an important protective role in the oxidative stress response that is induced during dehydration in the production of active dry wine yeasts.

2.3. Molecular characterisation of yeasts

Assessing gene expression levels is a key complementary analysis to the previously described cytological and physiological characterisation. On this regard, it is particularly relevant to evaluate expression dynamics of stress-marker genes, i.e. genes responsive to one or more stress[7] conditions, so as to better understand the hostile environmental conditions yeasts must endure once in the base wine and throughout the second fermentation. Besides providing important clues on yeasts' immediate, delayed and/or maintained stress responses (at the transcriptional level), this approach further allows to address yeasts' adaptation state, since many stress responsive genes code for products directly or indirectly involved in stress tolerance, *e.g.* heat shock proteins (HSPs) genes. It must be realized that base wines are complex and dynamic environments, in which yeasts are simultaneously and continuously exposed to a full mix of stresses. In order to clearly appreciate these multiple stresses and yeasts' specific and overall adaptation responses, expression analysis must be performed for a broad group of target genes, selected among the potentially most informative. Ideally, these genes should be induced only by certain stress conditions, which would be easily recognized. Though many genes respond to various stresses (Gasch *et al*. 2000; Causton *et al*., 2001), some are induced more specifically, and can therefore be considered markers of a type of stress.

Well-known stresses such as low temperature and high ethanol must be permanently withstood by sparkling wine yeasts; genes especially induced by these stresses should therefore be considered. Under laboratory growth conditions, the transcriptional response of *S. cerevisiae* to cold stress (10ºC) appears to be partially time-dependent, showing sequential changes during cell exposure (Sahara *et al*., 2002; Schade *et al*., 2004; Aguilera *et al*., 2007). Early responses (0-2 h) commonly include enhanced transcription of *OLE1*, encoding the sole *S. cerevisiae* delta-9 fatty acid desaturase. At later times, a number of typical general stress response genes (genes whose expression is induced by a variety of stress conditions) become up-regulated, such as *HSP12* and some other HSP genes. Genes associated with glycogen (*GSY1*) and trehalose (*TPS1* and *TPS2*) synthesis are also induced in a delayed response to cold stress. Other characteristic cold responsive genes, expressed at variable times upon exposure to low temperatures (10ºC or less), include *TIP1*, *TIR1* and *TIR2*, encoding members of the DAN/TIR family of putative cell-wall mannoproteins (Schade *et al*., 2004; Aguilera *et al*., 2007). As for ethanol stress, it has been reported to strongly induce a number of HSP genes such as *HSP12* and *HSP30*. Also *TPS1*, *TPS2* and *NTH1*, involved in trehalose metabolism, are up-regulated (Alexandre *et al*., 2001; Kaino and Takagi, 2008; Ding *et al*., 2009). More specific ethanol stress gene markers have not yet been described.

[7] Any environmental factor with an adverse effect on cell growth is considered a stress condition (Ivorra *et al*., 1999).

Additional stresses like oxidative and hyperosmotic, whose extent is unknown in second fermentations, should also be addressed. Oxidative stress occurs under oxic conditions, being caused by reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radicals or superoxide anions. ROS are generated by normal metabolic processes (respiration or β-oxidation of fatty acids) and exposure to pro-oxidants or heavy metals (Estruch, 2000). Though in-bottle second fermentations are mainly anaerobic processes, base wines carry some dissolved oxygen when bottled, which may cause ROS to arise. In *S. cerevisiae*, *GSH1*, whose product catalyzes the first step in glutathione synthesis, is induced by H_2O_2 and superoxide anions. H_2O_2 also induces *TRX2* and *TRR1*, coding respectively for a thioredoxin and a thioredoxin reductase, which also help protect cells against oxidative stress (Estruch, 2000). As for hyperosmotic stress, it is generally caused by high extracellular solute concentrations, which are responsible for a decrease in cell cytoplasmic water content, cell volume and turgor pressure. Ethanol may also reduce intracellular water in yeasts, causing this type of stress (Walker and Dijck, 2006). Transcription of both *GPD1* (coding a glycerol-3-P dehydrogenase) and *GPP2* (coding a glycerol-3-phosphatase) is especially increased under hyperosmotic stress. Their products are responsible for the synthesis of glycerol, the major compatible solute in yeasts (Estruch, 2000).

Expression levels of gene markers for nutrient limitation or starvation, *e.g.* nitrogen starvation, could be useful to determine as well. Nitrogen is necessary for the growth and sustained fermentative activity of yeasts, and its deficiency is a common cause of sluggish or stuck fermentations. Mendes-Ferreira *et al*. (2007) identified several genes as potential candidates for predicting nitrogen deficiency under winemaking conditions, among them *MSC1*, *XYL2*, *RTN2*, and *ODC1*, which are up-regulated in response to nitrogen limitation and starvation.

The transcriptional levels of the aforementioned genes, as well as of others properly identified beforehand and whose genetic sequence is known (at least partially), can be determined by Real-Time RT-PCR. This technique has become the method of choice to detect, quantify and compare expression (mRNA) levels of target genes (Bustin *et al*., 2005), largely due to its unmatched specificity, sensitivity and simplicity. It is based on cDNA (produced from mRNA by reverse transcription) PCR amplification and its real-time monitoring with fluorescent reporter molecules. A number of fluorophore technologies exist for the purpose, based in either intercalating dyes (*e.g.* SYBR Green) or specific probes (there are different types; *e.g.* TaqMan); depending on the technology, fluorescence signals are generated through different events (VanGuilder *et al*., 2008). Real-Time PCR reactions are carried out in special thermocyclers that perform successive measurements on the fluorescence signals, allowing to follow the production/accumulation of amplification products and to quantify them during each PCR cycle. The more copies of cDNA target there are at the beginning of the reaction, the fewer cycles are necessary to generate a number of amplicons that can be reliably detected and quantified. The PCR cycle number at which the fluorescence reaches a defined threshold value is defined as the threshold cycle, C_T, typically used to evaluate and compare expression levels. Real-Time RT-PCR gene expression analyses can be either of absolute levels (“exact” copy number of a specific mRNA in a sample) or relative levels (proportion of a specific mRNA in a sample when compared with the amount of that same mRNA in other sample), yet the latter is most commonly done (VanGuilder *et al*., 2008). For relative

quantification, the expression level of a target gene must be normalized with respect to one or more reference/endogenous genes, whose expression must remain constant under all experimental conditions for which sample comparisons are to be performed (Vaudano *et al*., 2009). This normalization is made for each sample separately. Normalization is vital to avoid quantification errors arising from differences in the initial RNA quantity and integrity or reverse transcription efficiency between samples.

Analysing expression levels of selected genes can be very useful, but it does not provide an overall view on gene expression and therefore on the large-scale transcriptional reprogramming that may take place in response to stress(es) and during adaptation. Through genome-wide expression analyses with microarrays, Rossignol *et al*. (2003) and Marks *et al*. (2008) effectively demonstrated the extensive (and dynamic) transcriptional changes that occur during vinification in must, and which are associated with stress conditions. A similar approach would be very valuable to more completely understand yeasts' adaptation mechanisms triggered during the second fermentation.

3. Objective of this thesis

As already explained, sparkling wine yeasts must cope with very hostile environmental conditions once inoculated in the base wine and during the whole second fermentation. Very low temperatures, i.e. 10ºC or less, are especially problematic and may cause second fermentations to slow down, suddenly stop or not start at all, even when yeasts are properly acclimatized. These temperatures, not infrequent in large underground cellars where sparkling wines are produced by the traditional method (*e.g.* in Champagne region), cannot be controlled due to the high costs involved. This situation poses particular problems to producers.

The work performed for this thesis had the objective of studying and understanding sparkling wine yeasts' physiological changes and adaptation behaviour under very low temperature (10ºC) in-bottle second fermentations. For this purpose, two sparkling wine yeast strains (*S. cerevisiae*) and two base wines were used in small scale in-bottle second fermentations at 10ºC, mimicking the actual industrial production conditions. Each strain was separately inoculated into each of both base wines, and in all four experimental conditions a multiparametric characterisation of yeasts was conducted throughout the fermentation process. This characterisation, broad and integrative, focused on a number of cytological, physiological and molecular properties of yeasts and had the purpose of thoroughly assessing the evolution and heterogeneity of their vitality and adaptation state. Parameters evaluated were: viability; fermentation kinetics; total proteins; trehalose; glycogen; neutral lipids; and expression levels of selected genes (*HSP12*, *GPD1*, *GSH1* and *TRX2*).

Results from this study may provide useful information for industrial sparkling wine yeasts' manufacturers, who may ultimately think of new adjustments that might be introduced in their large-scale production processes in order to produce yeasts better adapted to the harsh second fermentation conditions, most especially to low temperatures.

MATERIALS AND METHODS

1. Study outline

Two different sparkling wine yeast strains (*S. cerevisiae*), referred to as strains 1 and 2 for confidential motives, and two different base wines (Table 1), named wines 1 and 2 for the same reason, were used in small scale in-bottle second fermentations. Proenol, the company with whom a collaboration was established for this study, provided both yeast strains in an encapsulated form (as calcium alginate beads) for direct inoculation, and also both wines. Each strain was separately inoculated into each wine, therefore accounting for four independent experimental conditions.

Second fermentations were carried in 125 ml glass bottles, each filled with 100 ml of wine and inoculated with 0.5 g of encapsulated yeast beads (about 1.9-2.8 x 10^9 total cells). Bottles were capped with rubber stoppers and further sealed with metallic rings, to completely avoid gas exchanges and allow pressure to develop during fermentation. Incubations were performed at 10ºC, without stirring or light exposure, for a maximum of 85 days. A total of 58 bottles were used, 14 for each strain/wine condition and 2 as blanks (wine only), to control for contaminations.

Time points for sampling of yeasts and wine were as follows: immediately before inoculation (day 0), and then 24 h, 48 h, 12 days, 19 days, 26 days, 58 days and 85 days post-inoculation (time points 0 to 7). Before inoculation, 3 samples (replicates) of each yeast and wine were taken. At each time point after inoculation, and for each strain/wine condition, 2 bottles were opened for sampling, corresponding to 2 biological replicates. Wine samples were used for determination of glucose and glucose plus fructose levels, allowing to assess fermentation kinetics. Yeast samples were used to analyse several cell parameters: viability, total proteins, trehalose, glycogen, neutral lipids, and relative expression levels of selected genes (*HSP12*, *GSH1, TRX2* and *GPD1*).

Table 1. Characteristics of the base wines used in this study.

Base wine	Free SO_2 (mg/l)	pH	Ethanol (v/v)	Glucose plus Fructose (g/l)	Assimilable nitrogen (mg/l)
Wine 1	9.6	2.93	11.4%	28.02	88.2
Wine 2	9.6	3.13	11%	27.36	99.4

2. Yeast samples processing

For each replicate, immediately after sampling, encapsulated yeast beads were either: *1)* put in RNA*later*[8] solution (Ambion) and kept at 4ºC for at least 24 h (one week maximum) before being dissolved (see below); or *2)* promptly dissolved. In *1)*, two groups of beads were separately collected for total RNA (40 beads in 2 ml RNA*later*) and total protein (10 beads in 0.5 ml RNA*later*) extractions. In *2)*, a single group of beads (between 60 and 80) was promptly dissolved to produce a *free* yeast cell suspension, hereafter mentioned as GB (global) suspension, used in all further analysis. In all cases, encapsulated yeasts were freed from the calcium alginate matrix by being put in a proper bead-dissolving solution (confidential receipt, provided by Proenol) and vortexed for ~10

[8] RNA*later* permeates cells, stabilizing and protecting RNA. Proteins denature but are also preserved.

min. After alginate dissolution, yeast suspensions were centrifuged (12 min, 4000 rpm, 4ºC), ressuspended in 4 ml PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.76 mM KH_2PO_4; pH 7.4) and vortexed. This step was repeated in all cases, for complete removal of dissolved alginate and proper cell washing before further sample processing/analysis. GB suspensions were kept on ice at all times, to prevent substantial metabolic changes in cells before analysis.

3. Glucose and glucose/fructose determinations in wine

Glucose levels in wine samples were assessed with a commercial kit from NZYtech (D-Glucose, UV method). Using this kit, D-glucose in a sample is first converted to glucose-6-P in the presence of ATP, a reaction catalysed by an hexokinase, and then glucose-6-P is converted to D-gluconate-6-P in the presence of $NADP^+$, a reaction catalysed by a glucose-6-P dehydrogenase. The second reaction generates NADPH, the amount of which can be measured at 340 nm and is stoichiometric with the amount of glucose initially present. The kit protocol was adapted in order for glucose determinations to be performed in 96-well microtiter plates (using a plate reader) instead of cuvettes (using a spectrophotometer). The following changes were introduced: *a)* only one tenth of all solutions' volumes to be utilized in cuvette measurements were employed in 96-well microtiter plate determinations; *b)* D-glucose standard solution (kit solution 4) was always used for standard curve preparation, in which the following D-glucose final concentrations were used: 0 (blank), 0.02, 0.05, 0.10, 0.20, 0.40, 0.60 and 0.80 mg/ml; *c)* absorbance at 340 nm was measured in a plate reader (Anthos Zenyth 3100; Anthos Labtec Instruments). Every quantification reaction was performed in triplicate (standards) or duplicate (samples), i.e. three/two plate wells were used. Standards' average absorbance values were used to create a standard curve, from which glucose contents in wine samples were determined. Final results were expressed as mg of glucose per ml of wine. Glucose plus fructose levels in wine samples were determined at Proenol laboratories.

4. Viability assessment with methylene blue (MB)

Viability determinations with methylene blue (MB) (0.1% in H_2O) were performed for each GB yeast suspension, simultaneously with total cell concentration estimations. For the purpose, a small volume of GB suspension was diluted in PBS buffer in a 0.5 ml tube and MB was added in a 1:75 proportion. This mixture was briefly vortexed, incubated 2-3 min, vortexed again and then 10 µl of it mounted on a Neubauer counting chamber. Countings of at least 300 cells (to reduce counting errors) were made with 400x magnification, during 5-10 min. Cells (live and dead, and therefore total) were counted in five larger squares of the central counting grid (each with 16 smaller ones) starting from the top leftmost and ending on the bottom rightmost, proceeding diagonally. Cells overlapping right or bottom rulings were always counted, whereas cells on the left and top were not. Cells stained blue were considered dead, along with broken, shriveled and plasmolyzed cells. Budding cells were counted as one cell if the bud was less than one half the size of the mother cell. Viability was calculated as the ratio of viable (non-stained) to total cells.

5. Viability assessment with flow cytometry (FC)

Viability determinations with flow cytometry (FC) were also performed for each GB yeast suspension, except at day 85. A commercial kit for assessment of yeast viability by FC was used (Yeast control – Viability; Partec) and its protocol followed. This kit has two different staining reagents, the exact nature of which is unknown (not revealed by the company). One of them is a green fluorescence dye that apparently stains all cells (it may be, for example, SYTO 9; Molecular Probes, 2005), while the other is red fluorescent and stains "dead" cells (most probably PI, as its absorption spectra was determined and seemed to match). When both dyes are present in cells ("dead"), a reduction in green fluorescence is observed, supposedly because of fluorescence resonance energy transfer between them (Molecular Probes, 2005). A certain volume of GB suspension, with 10^6-10^7 total cells, was first diluted in PBS buffer for a 1 ml final volume. The kit staining procedure was applied on this dilution. FC analysis was performed in a CyFlow SL flow cytometer (Partec) with True Volumetric Absolute Counting capability, equipped with a blue solid-state laser (20 mW at 488 nm). Sheath fluid was filtered (0.22 µm) PBS buffer. Forward scatter detector (FSC) gain was set to 242 V, side scatter detector (SSC) to 213 V, green fluorescence detector (FL1) to 332 V (adjustable) and red fluorescence detector (FL3) to 452 V. Trigger was set on FSC and all compensation settings were at zero. Logarithmic amplification was always used. Flow rate was adjusted to keep total acquired events below 2000/s, and approximately 15000 yeast cell events were acquired for each sample. Yeast populations were identified on an FSC vs SSC dotplot and gated for presentation on an FL1 vs FL3 dotplot, where live and dead cells were identified. Cells with high green and low red fluorescences were considered live, while cells with high red and low green fluorescences were considered dead. FC viability was calculated as the ratio of live to total cells. All data was acquired and analysed with Partec FloMax software.

6. Trehalose and glycogen quantification (AQ)

For trehalose and glycogen quantification in cells (referred to as absolute quantification, AQ), a GB yeast suspension volume corresponding to 3 x 10^8 cells was collected to a 1.5 ml microtube. After centrifugation (5 min, 13000 rpm, 4ºC), cells were ressuspended in 250 µl Na_2CO_3 (0.25 M) and incubated for 4 h at 95ºC (permeabilization). Next, 150 µl acetic acid (1 M) and 600 µl sodium acetate (0.2 M, pH 5.2) were added and the mixture vortexed. Then, 490 µl were transferred to each of two new 1.5 ml microtubes, numbered 1 and 2 and used for trehalose and glycogen determinations respectively. To tube 1, 40 µl of trehalase at 0.64 U/ml (diluted from 2100 U/ml stock; Sigma) were added. To tube 2, 20 µl of amyloglucosidase at 31.2 U/ml (diluted from 300 U/ml stock; Sigma) were added. Both mixtures were homogenised by pipetting. Tube 1 was incubated overnight at 37ºC (for trehalose hydrolysis, which generates two glucoses) while tube 2 was incubated overnight at 57ºC (for glycogen breakdown, also generating glucose). The day after, both tubes were centrifuged (3 min, 13000 rpm). From each, 400 µl of supernatant were collected for glucose quantification. Glucose levels were determined in the same way as for wine samples. Final results were expressed as µg of trehalose per 10^7 cells (for trehalose) and µg of glucose equivalents per 10^7 cells (for glycogen).

7. Trehalose, glycogen and neutral lipids analysis with flow cytometry (FC)

Trehalose and glycogen contents in yeast cells were also analysed with FC. Neutral lipids were selected as an additional parameter to assess with this technique. For both trehalose and neutral lipids determinations, commercial kits were used (Yeast control – Trehalose and Yeast control – Neutral lipids; Partec) and their protocols followed. Each of these kits has a specific staining reagent, a fluorescent dye, not revealed by the selling company. The trehalose dye fluoresces green, while the neutral lipids dye fluoresces orange. Acriflavine (1 mg/ml in PBS buffer), which ends up fluorescing green, was used to stain intracellular glycogen. Yeast samples for FC analysis of trehalose, glycogen and neutral lipids were collected from GB yeast suspensions, approximately 10^7 cells being transferred to each of three 1.5 ml microtubes (one separate sample for each parameter analysis). These cells were centrifuged (5 min, 13000 rpm), ressuspended in ice-cold 70% ethanol for fixation, and stored at 4ºC for at least 24 h. Immediately before staining for FC analysis, fixed cells were centrifuged (5 min, 13000 rpm) and washed with 1 ml PBS buffer. After new centrifugation, washing, and final centrifugation, cells were suspended in 1 ml PBS buffer for staining. Intracellular trehalose and neutral lipids staining was performed following kit instructions. Each suspension for intracellular glycogen determination was stained with 10 µl acriflavine and incubated in darkness for 30 min at room temperature. All FC analysis were performed in a CyFlow SL flow cytometer (Partec) with True Volumetric Absolute Counting capability, equipped with a blue solid-state laser (20 mW at 488 nm). Green fluorescence detector (FL1) gain was set to 283 V for trehalose analysis or to 338 V for glycogen analysis. For neutral lipids analysis, orange fluorescence detector (FL2) gain was set to 323 V. All other FC parameters were set as described for FC viability determinations. Aproximately 15000 yeast cell events were acquired for each sample in all analysis. Yeast populations were identified on an FSC vs SSC dotplot and gated for presentation in either: *1)* a green fluorescence (FL1) histogram, for trehalose or glycogen content analysis, or *2)* an orange fluorescence (FL2) histogram, for neutral lipid contents analysis. These histograms show the frequency distributions of fluorescence intensities of stained cells. Higher fluorescence intensities stand for higher intracellular contents of the stained compound. In the case of trehalose and glycogen, average values of fluorescence intensities were used for comparisons with AQ determinations. The robust coefficient of variation (RCV) was used as a measure of dispersion to compare fluorescence distributions, in order to perceive yeast populations' heterogeneities for trehalose, glycogen and neutral lipids. RCV has the advantage of not being as skewed by outlying values as the CV (Shapiro, 2003). RCV values were determined with FlowJo software (Tree Star). Higher RCVs were considered to represent higher diversity in populations. Fluorescence histograms were also looked at for their shape, to try to identify relevant distribution properties like bimodality, suggestive of two subpopulations with differing contents of the stained compound.

8. Total protein extraction and quantification

Total protein extraction procedure was based on a protocol described by von der Haar (2007). Each yeast suspension for total protein extraction and quantification was centrifuged and cells ressuspended in 1 ml PBS buffer and transferred to a 1.5 ml microtube. Cell countings (in a

Neubauer counting chamber) were performed at this stage (usually totalizing 0.7-1.3 x 10^7). After new centrifugation, cells were ressuspended in 200 µl lysis buffer (0.1 M NaOH, 0.05 M EDTA, 2% SDS, 2% β-mercaptoethanol) and an equal volume of acid-washed glass beads (425-600 µm) was added. The mixture was then vortexed for 30 s and incubated at 90ºC for 10 min. Next, 5 µl acetic acid (4 M) were added and the mixture again vortexed (30 s) and incubated at 90ºC (10 min). After centrifugation (3 min, 13000 rpm, 4ºC), the supernatant was collected in a 2 ml microtube and mixed with 800 µl methanol, 200 µl chloroform and 600 µl Milli-Q water in a stepwise manner, briefly vortexing between each addition. This mixture was then centrifuged (5 min, 12000 rpm, 4ºC) and the upper phase carefully discarded (protein could be easily seen at the interphase). Methanol (1200 µl) was added and the mixture vortexed and centrifuged (5 min, 12000 rpm, 4ºC). Finally, the supernatant was removed and 400 µl PBS buffer were used to ressuspend the pellet (protein), homogenising completely.

Total protein concentrations were thereafter determined with the Biuret assay. This assay is based on the complexation of copper ions with proteins' peptide bonds at basic pH, which generates a blue colour that can be measured spectrophotometrically. A 5 mg/ml bovine serum albumin (BSA) stock solution in PBS buffer was used for standard curve preparation. Standard BSA solutions with the following concentrations were used (stock BSA dilutions done in PBS buffer): 0 (blank), 0.1, 0.25, 0.50, 1.0, 2.5 and 5 mg/ml. A 40 µl volume of each standard and protein sample was transferred in triplicate to wells in a 96-well microtiter plate, then 200 µl of Biuret reagent[9] were added and mixed. After 20 min incubation at room temperature, absorbance at 570 nm was measured in a plate reader (Anthos Zenyth 3100; Anthos Labtec Instruments). Standards' average absorbance values (from triplicates) were used to make the standard curve, from which protein levels in samples were determined. Final results were expressed as pg of protein per cell.

9. Gene expression analysis

9.1. Total RNA extraction and cDNA synthesis

Total RNA extraction procedure was based on protocols described by Kruckeberg *et al*. (2009) and Mannan *et al*. (2009). Each yeast suspension for total RNA extraction[10] was centrifuged and cells ressuspended in 1 ml Milli-Q H_2O and transferred to a 1.5 ml microtube. Once vortexed, the suspension was centrifuged again (5 min, 13000 rpm, 4ºC). Cells were ressuspended in 400 µl AE buffer (50 mM sodium acetate and 10 mM EDTA; pH 5.0) and 40 µl SDS (10%) were added. The suspension was vortexed and about 200 µl of acid-washed glass beads (425-600 µm) were added, plus 500 µl of acid phenol:chloroform (MB Grade, Ambion) preheated to 65ºC. The mixture was vortexed for 1 min then incubated at 65ºC for 5 min. This last procedure was repeated two more times. Next, the mixture was centrifuged (5 min, 13000 rpm) and the upper aqueous phase (containing RNA) transferred to a new 1.5 ml microtube. A second hot acid phenol:chloroform

[9] Biuret reagent was prepared as follows: 1.5 g copper sulfate were dissolved in 50 ml Milli-Q water; to this, 6 g potassium sodium tartrate were added, then 1 g potassium iodide; lastly, 300 ml NaOH 10% were added and final volume was brought to 1000 ml with Milli-Q water.

[10] In order to avoid RNAse contamination and RNA degradation, water and most solutions used in RNA extraction were previously treated with 0.01% DEPC, being kept at 37ºC overnight and autoclaved thereafter. Microtubes were also treated this way. Glass beads were subjected to 230ºC dry heat for 3 h. Filter tips were used at all times.

extraction (with 500 µl) was performed, followed by a chloroform:isoamilic acid (24:1 v/v) extraction with an equal volume to the collected upper phase. The final upper phase was transferred to a new 1.5 ml microtube and kept on ice for 2 min. A 1/10 volume sodium acetate (3 M, pH 5.0) was then added, as well as 1 ml ice-cold absolute ethanol to precipitate RNA. This mixture was incubated for 1 h at -20ºC and 15 min at -80ºC. It was then centrifuged (20 min, 13000 rpm, 4ºC) and the pellet (RNA) washed with 500 µl ice-cold 70% ethanol. After new centrifugation (10 min, 13000 rpm, 4ºC) the pellet (RNA) was left to dry at 37ºC for ~25–30 min. RNA was then ressuspended in 100 µl of Milli-Q H_2O and stored at -20ºC if not immediately needed. RNA quality and integrity were confirmed by agarose gel electrophoresis, running 5 µl of RNA samples on a 1% agarose gel in 0.5x TBE buffer, for 60 min at 90 V. RNA quantification was performed with the Quant-iT RNA assay kit (Invitrogen) in a Qubit fluorometer (Invitrogen), according to manufacturer instructions.

To remove contaminating genomic DNA from RNA samples, the Turbo DNA-*free* kit (Ambion) was used and its guidelines followed. For each RNA sample, 0.25 µg RNA were treated with 6 U of Turbo DNase in a 20 µl reaction volume in a 0.5 ml microtube, incubating for 60 min at 37ºC. Half the volume, 10 µl, was collected at the end of the process and used for reverse transcription (RT). DNAse treatments were performed in duplicate for each sample; the duplicate was later used as a negative control (for genomic DNA contamination) in real-time PCR amplification, not being subjected to RT. This control was referred to as non-template control (NTC). RT for first strand cDNA synthesis was performed in a 20 µl reaction volume with 200 U (1 µl) of SuperScript III Reverse Transcriptase (Invitrogen), following the exact product protocol. Oligo$(dT)_{20}$ (Invitrogen) were used as primers (1 µl), and RNaseOUT RNase Inhibitor (Invitrogen) applied (1 µl) as recommended. RT reaction was carried out for 60 min at 55ºC. The produced cDNA, to be used in quantitative real-time PCR for gene expression analysis, was diluted 10x (for a final 200 µl volume) and stored at -20ºC. NTCs were diluted to the same volume and also kept at -20ºC.

9.2. Relative gene expression analysis by quantitative real-time PCR

Quantitative real-time PCR was used to assess the relative expression levels of genes *ACT1*, *HSP12*, *GPD1*, *GSH1* and *TRX2*. Real-time PCR reactions were conducted in a StepOnePlus Real-Time PCR system (Applied Biosystems), and each amplification reaction was performed in a 20 µl volume, with 2 µl cDNA or control (NTC or blank, i.e. water), 0.2 µM of forward and reverse primers (final concentration) and 10 µl of Power SYBR Green PCR Master Mix (Applied Biosystems), which contains SYBR Green I for fluorescent labelling (it binds double-stranded DNA). All amplification reactions of a single time point were performed in the same 96-well plate. This included *ACT1*, *HSP12*, *GPD1*, *GSH1*, *TRX2* and *18S* (*RDN18*) amplifications for each cDNA sample of a time point, with *18S* being used as an endogenous/reference control. Different plates were used for different time points. No technical replicates were done (except when primer efficiencies were determined, see below). NTCs, always with *18S* primers, were used for all samples. Blank controls were applied in different wells of all plates, with *18S* or *ACT1* primers. The following amplification conditions were used: 95ºC for 10 min, then 40 cycles of 45 s at 95ºC, 45 s at 55ºC and 50 s at 72ºC. A melting curve analysis was always conducted at the end of the amplification cycles to confirm the specificity of each reaction. This was carried out by heating amplification products from

60°C to 95°C at 0.3°C/s and monitoring fluorescence decrease. Single peaks in melting curves' derivative plots were indicative of single amplicons in each reaction.

With the exception of *HSP12* primers, which were designed using the free PerlPrimer application (Marshall, 2004), all other primer pairs used in this study (for *18S, ACT1, GSH1, TRX2* and *GPD1*) were designed by Garre *et al*. (2010). Every primer was purchased from Invitrogen. Primers' specificity was initially tested by running their amplification products (obtained by traditional PCR) on agarose gel. For the amplification efficiency of each primer pair to be determined, an undiluted cDNA pool was made from undiluted cDNAs (i.e. not diluted after RT) of day 0 samples and used to produce a range of standard quantities for standard curve amplification experiments (in real-time PCR). Pool standards, obtained by serial dilutions, ranged 5 logs and were amplified in triplicate for accurate efficiency determinations, also allowing to assess technical reproducibility. Amplification efficiencies (Table 2) were calculated by StepOnePlus instrument software (StepOne software, Applied Biosystems) using the slope of the regression line in each primer pair standard curve. Efficiency differences were noteworthy in some cases (*e.g.* between *18S* and *ACT1*); yet, StepOnePlus software allows to correct expression levels for each target gene according to user-specified amplification efficiencies, therefore avoiding quantification errors. This correction can be applied when expression levels are determined with the comparative C_T method (see below).

Day 0 (before inoculation) was considered the reference point, i.e. all target genes' expression levels at the remaining time points (throughout second fermentations in both wines) were determined comparatively to the ones observed at time point 0. This was performed separately for each strain. Relative expression levels were calculated with StepOne software according to the comparative C_T method (also known as $\Delta\Delta C_T$ method; see Livak and Schmittgen (2001) for details), with corrections for amplification efficiencies. For the purpose, a simultaneous analysis of all amplification results was performed at the end of the study. During this analysis, a fixed threshold level for each gene was automatically defined by the software. Also, average expression levels at time point 0 (resulting from the three replicates) were considered the reference.

Table 2. Primers, amplicon sizes and amplification efficiencies for genes whose expression levels were followed in this study.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon size (bp)	Amplification efficiency (0-100%)
18S	TTGCGATAACGAACGAGACC	CATCGGCTTGAAACCGATAG	95	86.072
ACT1	CATGTTCCCAGGTATTGCCG	GCCAAAGCGGTGATTTCCT	51	93.310
HSP12[11]	AGTCATACGCTGAACAAGG	TTGGTTGGGTCTTCTTCAC	255	84.900
GPD1	GGTGAGATCATCAGATTCGG	CCTAGCAACCTTGACGTTTC	129	88.144
TRX2	GCTGAAGTTTCTTCCATGCC	GACTCTGGTAACCTCCTTAC	63	89.168
GSH1	CCGGACAAAAAGGATTCTCC	CGGAATACGCAGCGTTCTC	88	91.848

[11] *HSP12* primers were not designed following common recommendations (Applied Biosystems, 2001; Ambion, 2010) for smaller amplicon sizes in Real-Time PCR (amplicon length should be <150 bp), as they were initially intended for conventional RT-PCR. Nevertheless, despite the slightly lower amplification efficiency (certainly due to amplicon length), *HSP12* primers were used in this study without further detectable problems.

10. Statistical analysis

Two-sample homoscedastic Student's *t*-tests were performed for selected parameters at time point 0. Two-way analysis of variance (ANOVA) tests were conducted for selected parameters at all the remaining time points, factors being strain and base wine. Normality was assumed, as it could not be determined. Homoscedasticity was assessed with Hartley's test (Sokal and Rohlf, 1994). Under non-homoscedastic conditions, two-way ANOVA for ranks (Friedman ANOVA) was performed instead, after rank transformation of averages (Iman and Conover, 1989). For both *t*-tests and ANOVAs, Excel software was used. Principal component analysis (PCA) was performed with NTSYSpc software (version 2.20d; Exeter Software). The data matrix was structured with 32 samples, representing a single strain in a single wine at a single time point, and 15 variables (glucose in wine; glucose/fructose in wine; MB viability; FC viability; total proteins; AQ trehalose; FC trehalose; AQ glycogen; FC glycogen; FC neutral lipids; expression of *ACT1*, *HSP12*, *GSH1*, *TRX2*, and *GPD1*).

ATTENTION!

Due to confidentiality issues, absolute data values are not presented in this thesis and most results are instead shown as proportions relative to day 0 (prior to inoculation).

RESULTS AND DISCUSSION

1. Fermentation kinetics

Fermentation kinetics was assessed by following glucose and glucose plus fructose (GF) levels in wine (Figure 1). An initial fermentation delay of at least 2 days was observed in all strain/wine cases, except for strain 2 in wine 1, for which the GF levels had already decreased by 8% at 48 h post-inoculation (against 2% in average for other strain/wine conditions). By day 12, both strains were already actively fermenting in both wines, yet faster in wine 2, whose GF contents were by then considerably lower than in wine 1. In fact, at day 12, GF values were seen to highly significantly depend on the base wine ($p<0.001$), but not on the strain ($p=0.56$), also meaning that both strains were fermenting at similar rates. It should be mentioned that both wines had almost equal amounts of GF before inoculation (differing by only 0.66 g/l), as well as similar glucose contents (differing by 0.42 g/l) and therefore glucose/fructose ratios.

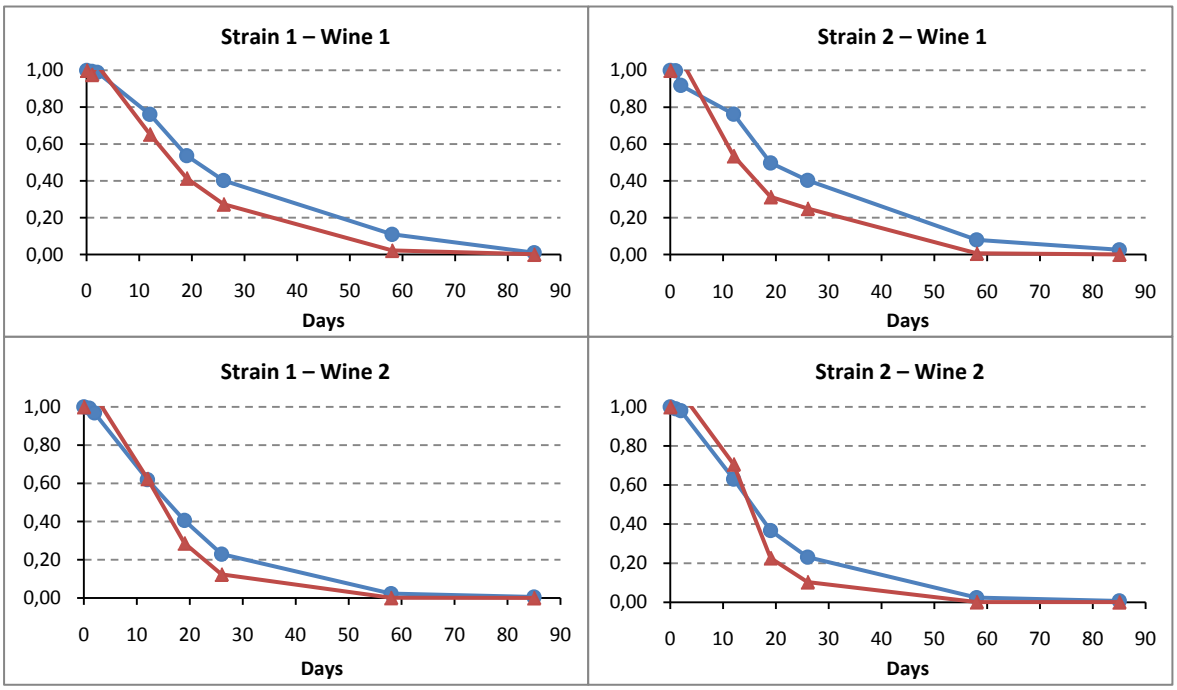

Figure 1. Evolution of the fermentation process. Presented values are proportions relative to day 0 (before inoculation) of glucose (red) and glucose plus fructose (blue) contents in wine. Averages for replicates (2) were used in all time points.

Fermentations ended earlier in wine 2, regardless of the strain. By day 58 after inoculation, virtually all fermentable sugar (GF) in wine 2 had already been consumed by both strains, with only residual levels of fructose still left. On the other hand, wine 1 still had moderate quantities of

fructose in both conditions, with GF amounting more than 2 g/l. Nevertheless, at day 85, both glucose and fructose were practically depleted in all cases, meaning all fermentations had finished.

Overall, both strains had quite comparable fermentative behaviours, irrespective of the wine. In wine 1, strain 2 showed a reasonably faster consumption of glucose between days 2 and 19 when compared to strain 1; yet, no other noteworthy dissimilarities were observed for sugar consumption kinetics. In fact, from days 12 to 85, both glucose and GF levels were almost always determined not to significantly depend on the strain ($p>0.05$). Only three exceptions were observed, two of which had associated a very ($p=0.008$; GF levels at day 58) or highly ($p<0.001$; glucose levels at the same day) significant interaction effect for strain and base wine. However, these exceptions cannot be considered relevant in the global analysis.

Globally, these results indicate that: *a)* both strains had very similar fermentative capacity in each wine; *b)* both adapted faster and better to wine 2. Together, these observations suggest that both strains were similarly affected by each wine chemical medium, with wine 2 being more amenable, and also that their physiological state at the time of inoculation was somewhat comparable. Wine 2 had slightly lower initial ethanol contents, higher pH and higher assimilable nitrogen levels than wine 1 (Table 1), which may have accounted for the differences seen between wines.

According to Ribéreau-Gayon *et al.* (2006), a second fermentation at 11-12ºC may take one month to finish, sometimes longer, whereas Jackson (2000) states it can last approximately 50 days at 11ºC. The second fermentations described on this thesis were performed at a lower temperature, 10ºC, which is expected to slow cell metabolism and delay the onset of fermentation even more. Most importantly, this lower temperature is thus expected to be responsible for an even bigger reduction in the global fermentation rate (Jackson, 2000), and may ultimately cause fermentations to abruptly end. In fact, and although none of the performed second fermentations became stuck, they took considerable time to successfully finish: around 2 months in wine 2, and a longer period (between 2 and 3 months) for wine 1.

2. Viability

Viability was determined with two methods, methylene blue staining (MB) and flow cytometry (FC). Obtained results are presented in Figure 2.

Good correlation between MB and FC

Linear regression calculus demonstrated an excellent correlation ($r\approx0.97$; $n=54$) between the full set of viability values obtained with both methods, MB and FC. Nevertheless, this correlation was slightly lower ($r\approx0.92$) if only values below 80% (viability percentage) in at least one method were considered ($n=25$). Still, these correlations are considerably higher than the ones achieved by Boyd *et al.* (2003) with brewing yeasts. These authors obtained an $r=0.87$ for all samples analysed ($n=179$) and only $r=0.39$ for samples with less than 85% viability by both methods (MB and FC). It must be noted, however, that Boyd and colleagues used a fluorescent oxonol dye to assess brewing yeasts' viability with FC, a procedure that differs from the one described on this thesis. Oxonols are membrane potential probes; being lipophilic but negatively charged, they tend to

remain outside cells with interior-negative membrane potentials (typical of live cells with intact membranes), only accumulating to a significant extent inside cells with damaged membranes or in which membrane potential is somehow absent (these cells are considered non-viable or dead) (Shapiro, 2003). Although the "membrane integrity" criterion is somewhat involved, oxonol may give slightly different staining patterns from PI (Deere *et al.*, 1998; Attfield *et al.*, 2000), one of the stains that is thought to be part of the commercial kit used in this thesis' work. Obviously, the differences between the aforementioned correlation values may also arise from other variations in experimental procedures, which may influence the viability values achieved with either FC (*e.g.* procedure for sample preparation and staining (Watson, 1992) or MB (*e.g.* amount of time samples spend with MB; counting of weakly stained cells; total number of cells counted). It must also be considered that for the work described on this thesis, only 54 different samples were analysed for viability, considerably less than the 179 samples examined by Boyd *et al.* (2003).

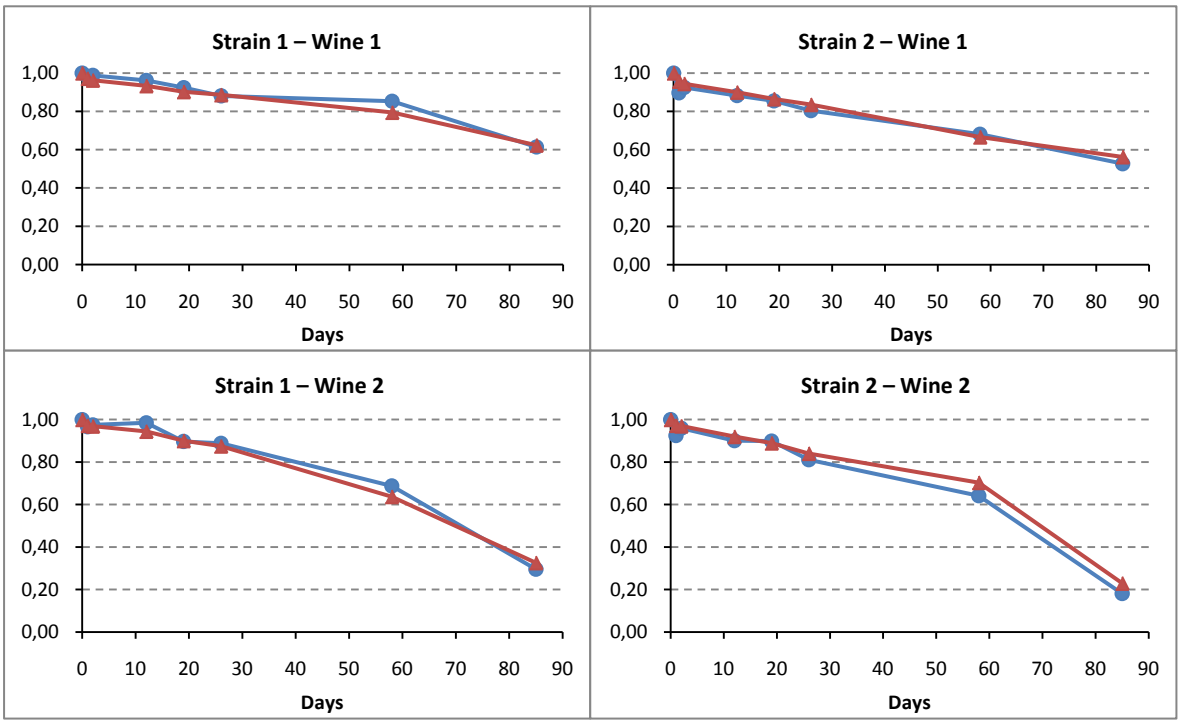

Figure 2. Evolution of yeasts' viability throughout the fermentation process. Presented values are proportions relative to day 0 (before inoculation) of FC viability (red) and MB viability (blue) percentages. Averages for replicates (3 at time point 0, 2 at the remaining time points) were used.

MB overestimates viability

Despite the high global correlation between MB and FC viability values observed in this study, MB determinations generally gave slightly higher percentages of live cells than FC. This happened for 39 out of the 54 samples analysed (≈72%). These results again oppose to the ones achieved by Boyd *et al.* (2003). These authors mention, however, that MB has been reported elsewhere to overestimate viability (probably when compared with regular plate culture assessments).

Viability evolution

The following considerations will be based on viability values obtained with FC. MB results may, nonetheless, allow for identical observations/conclusions. FC viability determinations were not

performed at the last time point (day 85); in this case only, the correspondent values were estimated from the FC-MB linear regression equation (based on the overall average viability values for each method).

As shown in Figure 2, at day 26 after inoculation, when relatively high GF amounts had already been consumed (more in wine 2; see Figure 1), both strains still had a fairly high viability proportionally to day 0 (before inoculation). Moreover, each strain had by then a very similar viability in both wines, and only a small proportional difference was observed between both strains, with strain 1 showing less loss of viability from day 0 (average proportion for both wines: 0.89) than strain 2 (average proportion: 0.84). This suggests an overall good capacity of both strains, especially strain 1, to successfully withstand and adapt to the difficult environmental conditions in both wines, which were not remarkably different as to affect viability in a very different way. In fact, except for day 85 (see below), no evidence was found of viability being significantly influenced in a different way by each wine environment ($p>0.05$ in all cases). Nevertheless, as previously noted, fermentations in wine 2 performed faster, which means this wine had a better global environment for fermentations to be carried out. By day 85, both strains were considerably reduced in their viability, most especially in wine 2, certainly because the available fermentable sugar had been depleted earlier in this wine. In fact, sparkling wine yeasts viability is expected to quickly decrease once the second fermentation finishes (Jackson, 2000).

An important consideration is that strain 1 had a very significantly higher absolute viability than strain 2 before inoculation (9.83% higher; $p=0.006$), and which remained higher throughout the second fermentations (data not shown). Except for day 85, differences in viability values were in fact seen to be significantly dependent on the strain ($p<0.01$ in all cases). Nevertheless, it must also be taken into account that strain 2 was inoculated in significantly higher total cell numbers than strain 1 (with 38.5% more cells; $p=0.02$), because each bead of the first had a higher total number of cells than each bead of the latter (data not shown), and also because an approximately equal number of beads of each strain was used as inoculum (corresponding to 0.5 g in total weight). This may explain, at least partly, the comparable fermentative performance of both strains (already discussed), with the lower viability of strain 2 being compensated by its higher initial inoculum.

3. Total proteins

As displayed in Figure 3, from before inoculation to 24 h post-inoculation a slight decrease in total protein amounts per cell was detected in all strain/wine cases, except for strain 2 in wine 2 (for which there may have been some experimental error during total protein quantification). This might have been caused by a substantial degradation of denatured and/or misfolded proteins, perhaps originated during yeast dessication and/or rehydration in the base wine. Proteolysis of unnecessary proteins, associated with the adaptation to the base wine environment, could also explain the observed results. After the initial decrease, total protein contents continuously rose until at least between days 19 and 26. This happened in all conditions, though at various rates, and reflects an active cell metabolism. From this time period until day 85, total protein levels steadily diminished, which was probably a consequence of the lowering viability (Majara *et al.*, 1998) and induction of autophagy due to the progressively scarcer sugar contents in wine (Cebollero and Gonzalez, 2006).

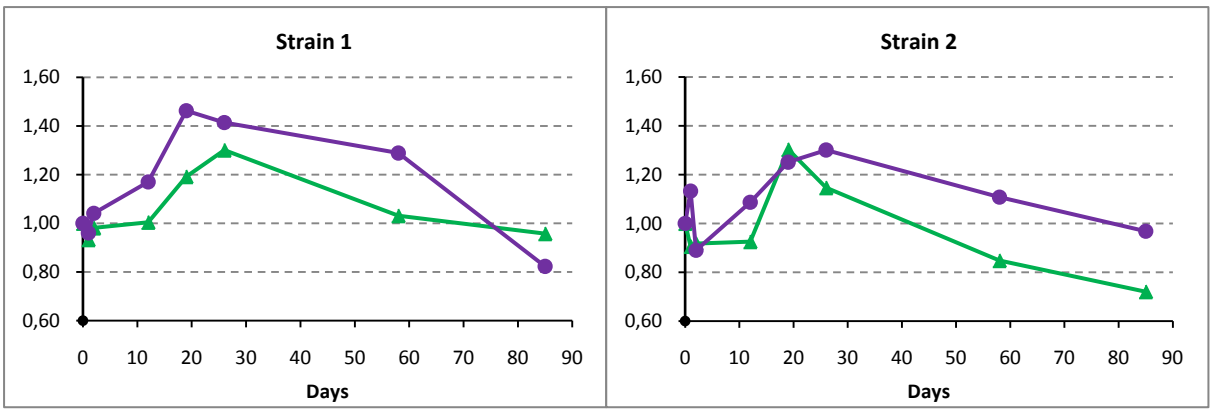

Figure 3. Evolution of yeasts' total protein contents during the fermentation process. Presented values are proportions relative to day 0 (before inoculation) of total protein levels in base wine 1 (green) and base wine 2 (purple) fermentations. Averages for replicates (3 at time point 0, 2 at the remaining time points) were used.

4. Trehalose

Intracellular trehalose contents were analysed by two methods: *a)* they were enzymatically quantified after extraction from cells, which provided an average absolute value for trehalose levels in each cell (absolute quantification, AQ); *b)* they were also examined by flow cytometry (FC), with the purposes of assessing population heterogeneities in trehalose contents and verify if both methods could provide comparable and correlated results.

Trehalose dynamics (AQ)

According to AQ determinations, trehalose levels increased considerably in the first 24 h but then diminished until day 2, in every strain/wine condition (Figure 4). This is an interesting observation. It seems that shortly after inoculation yeast cells promptly synthesised and accumulated substantial amounts of trehalose, but also quickly degraded plenty of it afterwards. This was probably an adaptive/stress response to the harsh base wine conditions, particularly to the high ethanol levels. Ethanol stress was reported to induce genes *TPS1*, *TPS2* and *NTH1* and cause a rapid increase in intracellular trehalose (Alexandre *et al.*, 2001; Kaino and Takagi, 2008). Those three genes code for Tps1p (Tre6P synthase), Tps2p (Tre6P phosphatase) and Nth1p (neutral trehalase), respectively. The fact that ethanol stress simultaneously induces genes whose products are involved in trehalose synthesis and degradation may allow yeast cells to rapidly adjust their trehalose levels to properly assist molecular chaperones, particularly HSPs (whose genes are also induced by ethanol stress), in protein refolding (Alexandre *et al.*, 2001). Trehalose is able protect native proteins from denaturation, replacing water molecules at their surface; however, especially if at high levels, trehalose also suppresses aggregation of already denatured proteins in a way that may hinder their refolding by chaperones. Thus, the synergy between trehalose and HSPs in stress tolerance may only be effective if trehalose accumulation in response to stress is followed by its degradation (François and Parrou, 2001). This may perhaps explain the very quick accumulation and subsequent degradation of trehalose observed during the first 48 h, being a dynamic response to ethanol shock. It should be emphasized that the capacity of cells to quickly accumulate (and degrade soon after) trehalose in response to ethanol stress (or other stresses) is linked not only to gene expression induction, but also and certainly to a great extent to the amount of enzymes

involved in trehalose metabolism cells already contain before being exposed to the stress, and which may be post-transcriptionally regulated (François and Parrou, 2001). Obviously, substrate availability (glucose) is also relevant for the synthesis step.

Maximum trehalose contents (AQ) were detected at days 19 or 26 in all strain/wine cases (Figure 4). The progressively more stressful wine environment (with increasing ethanol and pressure, and sustained inhibitory action of other stress factors like low temperature) may have been responsible for triggering an enhanced synthesis and accumulation of trehalose, which reached its highest levels around those two time points. A regular and cyclic trehalose turnover would nevertheless be expected to happen, for reasons mentioned above. Alternatively, or concurrently, trehalose may have been accumulated for its purpose as reserve carbohydrate, in response to lowering levels of externally available sugars (a stress condition as well, nonetheless). After reaching its peak, trehalose contents continuously decreased (with few exceptions) until day 85. This decline was most prominent for both strains in wine 2, with trehalose levels reaching very low values at day 85. Day 85 was in fact the only time point at which intracellular trehalose contents were seen to significantly depend on the base wine environment ($p=0.001$), with no effect being associated with the strain ($p=0.12$). For both strains in wine 2, trehalose was certainly extensively used as an alternative carbon and energy source, providing an answer to sugar starvation, which happened only later in wine 1. However, using up trehalose probably contributed to lower stress protection, especially from ethanol and low temperature, thereby promoting yeast cell death – at day 85, viabilities in wine 2 were very low (Figure 2).

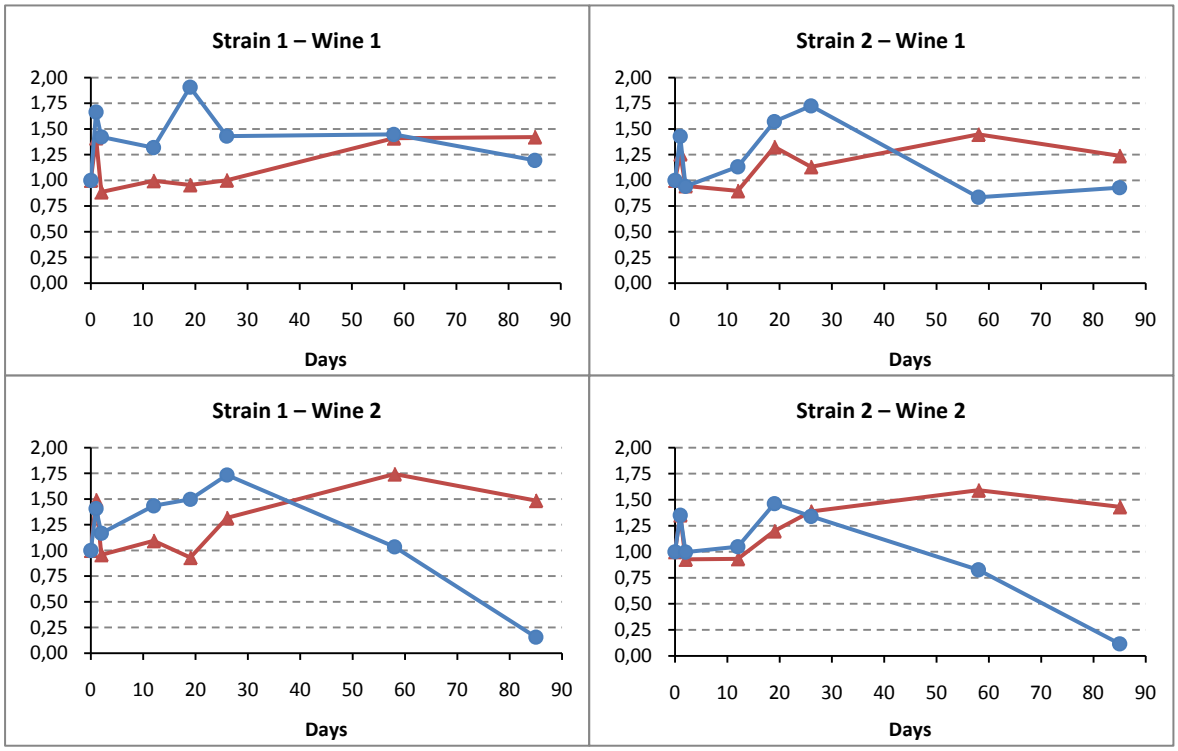

Figure 4. Evolution of yeasts' trehalose contents throughout the fermentation process. Presented values are proportions relative to day 0 (before inoculation) of AQ trehalose (blue) and FC trehalose (red) levels. Averages for replicates (3 at time point 0, 2 at the remaining time points) were used. FC analysis was based on averages of fluorescence intensities.

Higher trehalose levels in strain 1

Strain 1 had slightly higher trehalose levels (AQ) than strain 2 before inoculation (data not shown). The difference, though not statistically significant (p=0.37), could be one reason (certainly among others) for the higher viability of strain 1 prior to inoculation. The industrial production of active dry yeast forms, including encapsulated yeasts, involves their propagation and dehydration (Garre *et al.*, 2010; Proenol, 2010c), and trehalose may be a fundamental protective agent against desiccation effects, helping to stabilize and preserve yeast membranes' structure (François and Parrou, 2001). In fact, some studies reveal that intracellular trehalose is an important factor in improving dried yeasts viability and vitality (François and Parrou, 2001; Walker and Dijck, 2006). Hence, because of its higher trehalose contents, strain 1 may have better tolerated the dehydration step, thus maintaining a higher viability than strain 2. Throughout the whole second fermentation in both wines, strain 1 also continuously kept, to various extents, higher trehalose levels than strain 2 (the single exception being observed at day 26 in wine 1; data not shown). At several time points (days 2, 12, 26 and 58) trehalose levels were in fact seen to very significantly depend on the strain ($p<0.005$ in all cases), though an interaction effect was also observed in half the cases (days 2 and 26). The consistently higher trehalose contents of strain 1 could perhaps justify, at least partially, its greater viability also throughout the experiment.

Comparison with trehalose metabolism during must fermentations

To this date, no scientific reports covering trehalose metabolism during a second fermentation were yet published. Some studies during alcoholic fermentations in grape must have been performed (Gimeno-Alcañiz *et al.*, 1999; Roustan and Sablayrolles, 2002; Novo *et al.*, 2003; Rossignol *et al.*, 2003; Novo *et al.*, 2005), but they should not be considered a reference for in-bottle second fermentation observations. Must fermentations take place in very different conditions from base wine fermentations: high ethanol contents are not present since the very beginning; fermentable sugar levels are considerably higher (around ten times more); fermentation temperatures are typically higher (more yeast-friendly); among other certainly relevant differences. Therefore, yeasts' adaptation responses and overall behaviour will certainly follow a different path in each condition (must or base wine). Because of this, comparisons should be carefully done, to avoid erroneous interpretations.

In must fermentations, trehalose is commonly reported to reach maximum levels at the stationary phase of growth (Roustan and Sablayrolles, 2002; Rossignol *et al.*, 2003; Novo *et al.*, 2005). The highest trehalose levels observed in this thesis' work cannot be assigned to a specific growth phase, because no clear evidence of substantial yeast cell multiplication was found during the whole experiment (data not shown). A variable loss of cells during sample processing, which involved several steps, may have been a reason for cell multiplication not to be noticed. Plus, total cell counts in a Neubauer counting chamber are subject to some error. It is probable however, that cell multiplication did *really* not occur at a considerable degree as to be perceived, due not only to the very stressful base wine conditions, but also and mostly to the physical hindrance posed by the alginate matrix, which may have halted cell proliferation. Despite the absence of net growth, results achieved in this work can be compared to some observations during must fermentations, such as

the ones by Gimeno-Alcañiz *et al.* (1999). These authors saw a large decrease in trehalose levels when glucose was almost depleted, and also that complete trehalose consumption, occurring a few days after glucose exhaustion, led to rapid loss of viability. These results are analogous to the ones in this thesis' work, especially for wine 2, as already noted above. However, others exist that are completely different. For example, Novo *et al.* (2003; 2005) observed a sharp decrease in trehalose levels at the beginning of vinifications in must, at different temperatures and nitrogen availabilities.

AQ and FC relationship

No linear correlation was found ($r \approx 0.20$; $n=62$) between the full set of trehalose values obtained with AQ and FC (data not shown), despite the fact that common trends in the evolution of trehalose contents were sometimes observed between AQ and FC analysis (Figure 4), *e.g.* from day 0 to day 2 in all strain/wine cases and from day 12 to day 19 in strain 2 (both wines).

FC trehalose analysis was performed with a commercial trehalose staining reagent whose nature is not revealed by the selling company. Unfortunately, references to trehalose staining for FC seem to be virtually nonexistent in the scientific literature. Chlup *et al*. (2008), one of probably very few groups who actually reported the study of trehalose with FC (in brewing yeasts), used concanavalin A (ConA) to stain the disaccharide. This dye may actually be the commercial trehalose staining reagent that was used. However, ConA can react not only with trehalose, but also with any non-reducing α-D-glucose and α-D-mannose moieties, which may be present in other cell molecules such as glycoproteins (Worthington Biochemical Corporation, 2010). As a nonspecific stain, ConA may be responsible for inaccurate FC estimations of trehalose levels, associated with excess fluorescence intensities. This nonspecificity could perhaps explain, for example, the FC trehalose values that were observed at days 58 and 85 in both strains in wine 2 (Figure 4). In these cases, FC values were substantially higher than at day 0, which contrasted markedly with AQ assessments, especially at day 85. Nonspecific staining in FC could therefore be one reason for the lack of linear correlation between AQ and FC results in trehalose analysis.

In AQ, intracellular trehalose contents were determined by indirect quantification of glucose in cell extracts after trehalose hydrolysis with trehalase. It should be noted that no negative/blank controls were used, i.e. cell extracts not subjected to trehalase digestion (but otherwise processed and analysed according to the quantification protocol). Negative controls would have allowed a more precise assessment of intracellular trehalose levels, which would be properly corrected not to include intracellular free D-glucose and glucose-6-P, both quantified with the commercial kit that was used. It is thus possible that trehalose contents were variably overestimated by AQ at different times during the fermentation process, which might have contributed to the lack of correlation between both methods.

Population heterogeneities in trehalose levels

Despite the absence of linear correlation between AQ and FC for trehalose, FC data was further examined so as to assess yeast populations' heterogeneities in trehalose levels throughout the second fermentations. For this purpose, FC frequency distributions' robust CVs (RCVs) were determined and analysed. The distributions' shapes, as represented in histograms, were also considered and compared (histogram analysis was performed by straightforward observation only).

Figure 5A depicts the evolution of trehalose RCVs throughout the second fermentation for all strain/wine cases. It can be seen that RCVs changed to a variable and sometimes substantial extent between time points, in all strain/wine cases, and this suggests that all yeast populations under study had a dynamic/changeable heterogeneity in trehalose content during the second fermentation. Interestingly, each strain had associated to it a similar RCV evolution in both wines, which may indicate that both wines did not influence trehalose content heterogeneity very differently. Higher RCVs, related to higher overall heterogeneities, were observed in strain 1 (both wines), especially from day 2 onwards. Figure 6A shows selected histograms of FC trehalose distributions, representing both strains in wine 1 (results for wine 2 were similar). Considering each strain separately, no large differences were seen in histograms' shapes throughout the second fermentation process. Yet, distributions are noticeably different between both strains, with strain 1 showing what appears to be a second subpopulation with higher trehalose content, more evident at some time points (*e.g.* day 58) than others (*e.g.* day 2). Due to time limitations and other analysis priorities, trehalose heterogeneity was not further explored, and the potential relevance of these results not addressed.

5. Glycogen

As with trehalose, intracellular glycogen contents were analysed by two methods: *a)* they were enzymatically quantified after extraction from cells, which provided an average absolute value for glycogen content in each cell (AQ, in glucose equivalents); *b)* they were also examined by flow cytometry (FC), with the purposes of assessing population heterogeneities in glycogen levels and verify if both methods could provide comparable and correlated results.

Comparison between AQ and FC: glycogen dynamics

A weak linear correlation was observed ($r \approx 0.52$; $n=62$) between the complete set of glycogen values obtained with AQ and FC methods (data not shown). Even so, common trends between AQ and FC determinations were quite frequently seen in all strain/wine conditions (Figure 7). Between days 0 and 19, FC revealed a generalized continuous increase of glycogen in yeast cells. This global tendency was also observed in AQ assessments, though this last method had associated to it a few occasions in which glycogen levels actually diminished (*e.g.* strain 1 in wine 2, between days 12 and 19). From day 19 to day 26, FC glycogen levels dropped suddenly in all strain/wine conditions; yet, this directional switch was not observed with AQ, for which glycogen contents increased in all cases (to variable extents). In AQ, intracellular glycogen contents were estimated by indirect quantification of glucose in cell extracts after glycogen hydrolysis with an amyloglucosidase. As in the case of trehalose, for which the same glucose quantification kit was used, no negative/blank controls were performed in glycogen determinations (controls without amyloglucosidase treatment). Thus, in reality, AQ assessments of glycogen included not only glucose derived from the polysaccharide, but also pre-existent intracellular glucose and glucose-6-P. This may somehow explain the apparently contradicting results of both methods between days 19 and 26. During this period, substantial amounts of glycogen may have been degraded, and the resulting free glucose, much of it perhaps not immediately consumed, could have accumulated in

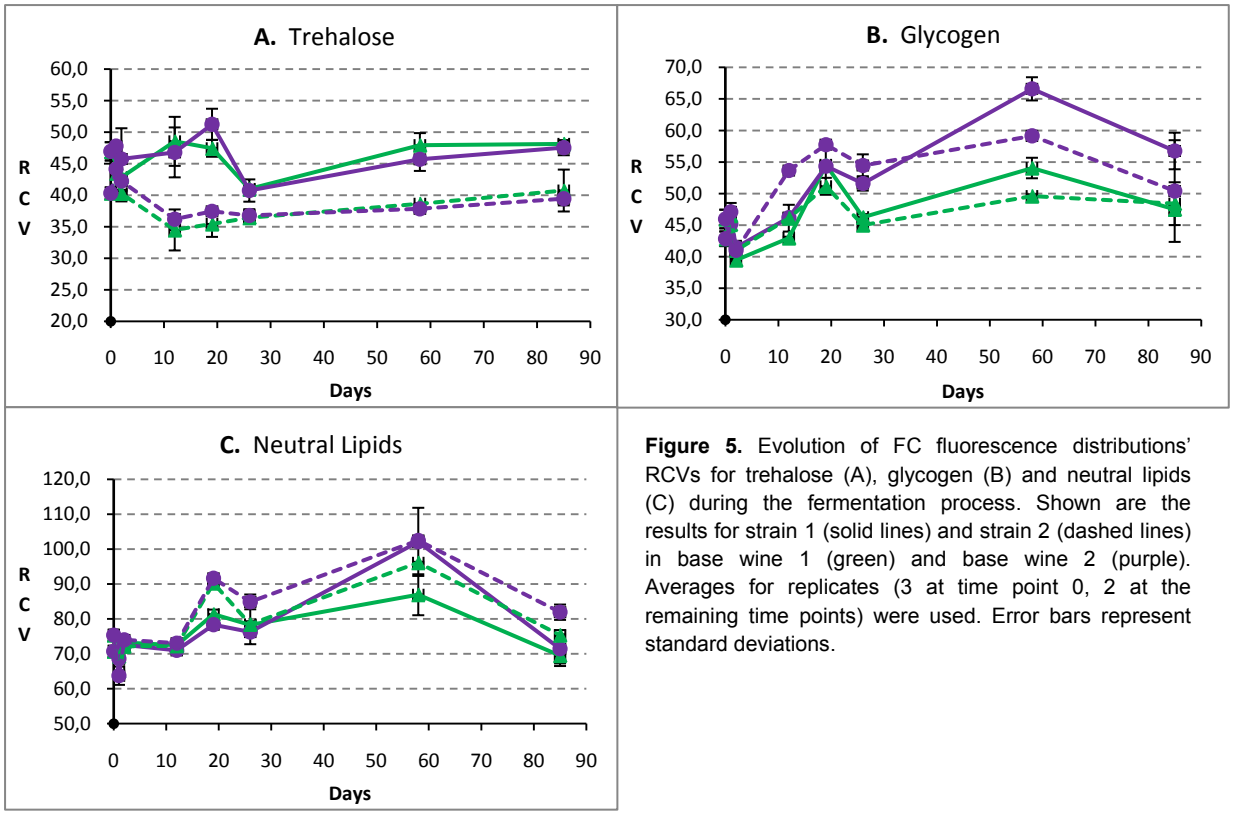

Figure 5. Evolution of FC fluorescence distributions' RCVs for trehalose (A), glycogen (B) and neutral lipids (C) during the fermentation process. Shown are the results for strain 1 (solid lines) and strain 2 (dashed lines) in base wine 1 (green) and base wine 2 (purple). Averages for replicates (3 at time point 0, 2 at the remaining time points) were used. Error bars represent standard deviations.

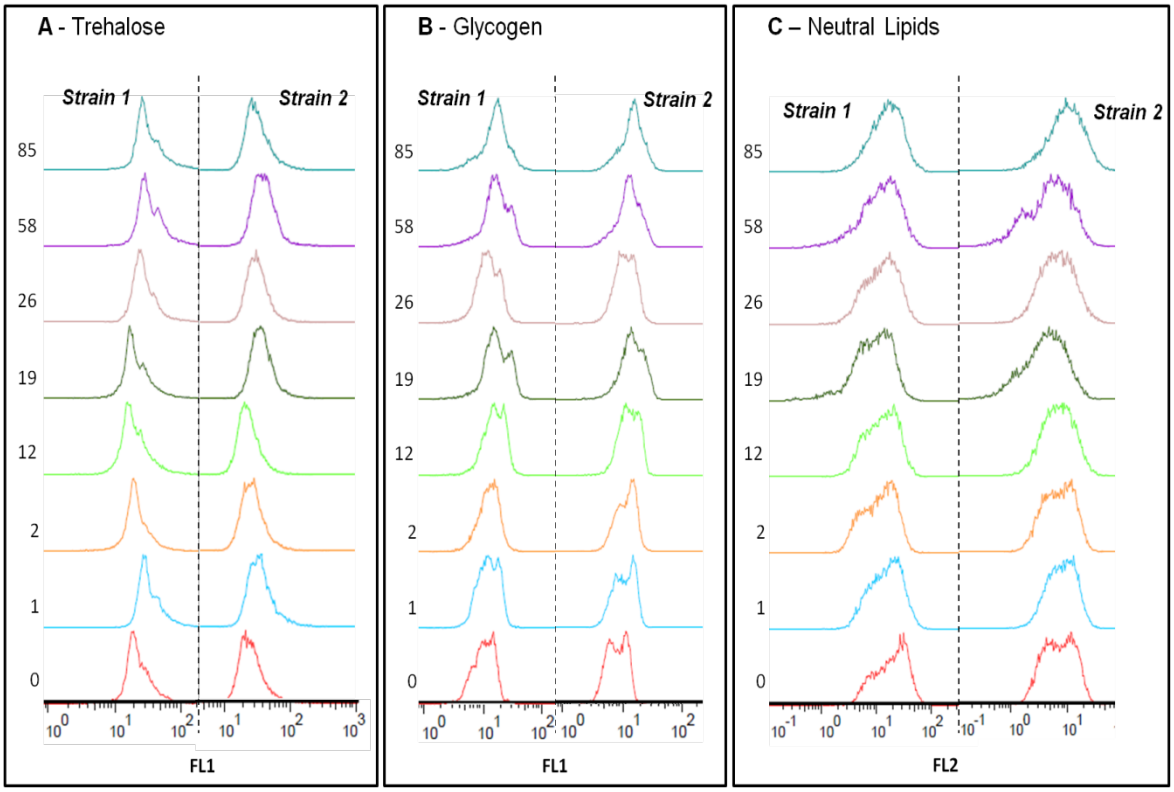

Figure 6. Selected FC histograms, corresponding to trehalose (A), glycogen (B) and neutral lipids (C) analysis. In A, B and C, every time point (indicated as days post-inoculation) is associated with two histograms, which represent one replicate of each strain. Depicted results refer exclusively to fermentations in wine 1.

cells. All this glucose would be detected by the AQ method and considered as originating from glycogen, due to the absence of negative controls. However, in the same circumstances, FC values for glycogen would diminish, because of the supposed specificity of acriflavine for the polyssacharide. AQ blanks would almost certainly clarify these results, and should therefore be used so as to avoid analogous situations.

Except for strain 2 in wine 1, AQ revealed maximum intracellular glycogen levels at day 58. FC values also increased between days 26 and 58, yet not achieving the highest results overall. Although AQ peaks are observed at day 58, glycogen probably reached its maximum levels earlier, especially in wine 2 (both strains), in which both glucose and fructose were already depleted at that point in time. It is thus likely that glycogen was already being consumed by then, along with trehalose, preventing starvation. Between days 58 and 85, as expected, intracellular glycogen was seen to decrease considerably; this indeed demonstrated its use as a carbon and energy reserve under the nutrient exhaustion conditions at hand. However, this decrease was not verified for strain 2 in wine 1, for which neither AQ nor FC had lowering values.

Despite the differences between AQ and FC determinations, both methods evidenced a similar pattern of glycogen synthesis and degradation between all strain/wine cases throughout the second fermentation. According to AQ, strain 2 in wine 1 was the only exception to the common pattern, notably from day 26 onwards. Overall, glycogen accumulation was not seen to be commonly nor consistently (for at least two sequential time points) dependent either on strain or on base wine ($p>0.05$).

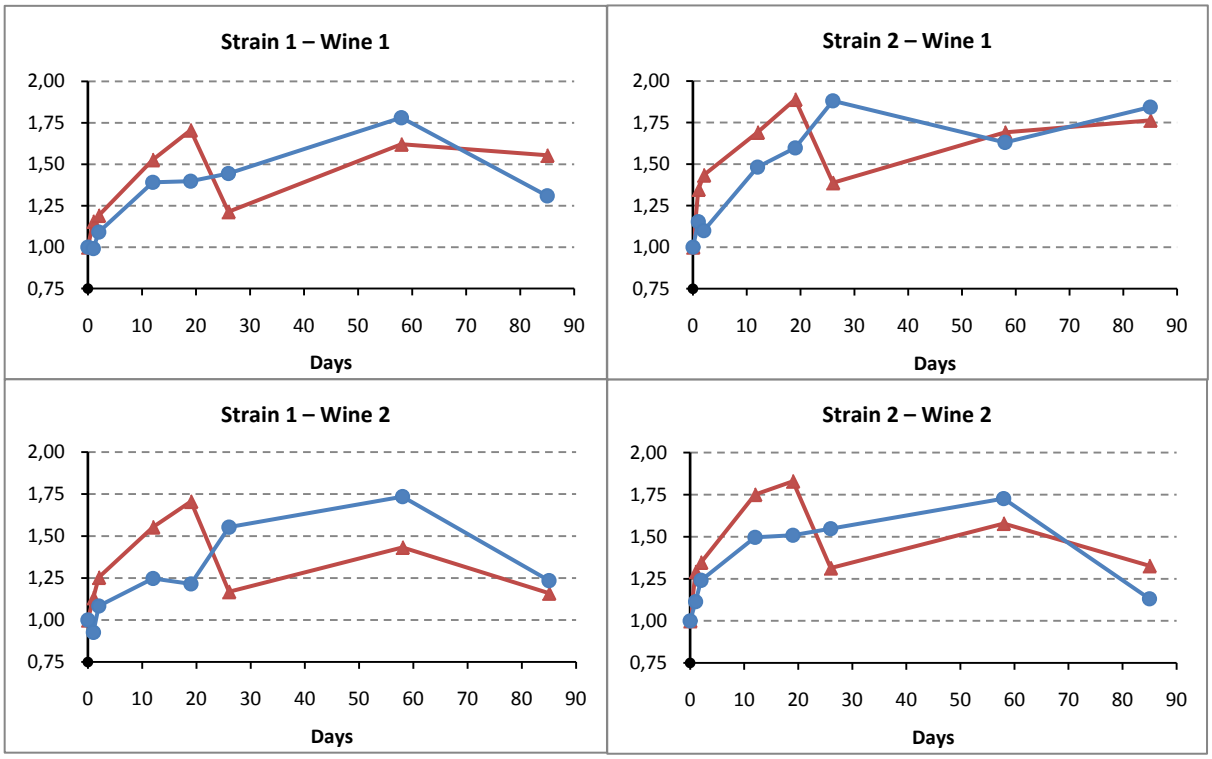

Figure 7. Evolution of yeasts' glycogen contents during the fermentation process. Presented values are proportions relative to day 0 (before inoculation) of AQ glycogen (blue) and FC glycogen (red) levels. Averages for replicates (3 at time point 0, 2 at the remaining time points) were used. FC analysis was based on averages of fluorescence intensities.

Comparison of glycogen levels between strains

Before inoculation, strain 1 had significantly higher glycogen levels than strain 2, according to AQ ($p=0.01$; data not shown). These higher contents in strain 1 were also verified with FC (no statistic test was performed). As in the case of trehalose, this may have contributed for the superior initial viability of strain 1 (Pretorius, 2000). Although FC values indicate that strain 1 maintained higher glycogen levels throughout the whole second fermentation in both wines, AQ results allow for different conclusions, showing that both strains achieved higher glycogen contents at different times in both wines (data not shown).

Glycogen metabolism during must fermentations

As with trehalose, there are no reported studies on glycogen metabolism in a second fermentation. During alcoholic fermentations in grape must, glycogen reaches highest levels at the stationary phase of growth, similarly to trehalose (Roustan and Sablayrolles, 2002; Rossignol *et al*., 2003). As previously explained, this cannot be assessed for this thesis' work.

Population heterogeneities in glycogen levels

To assess yeast populations' heterogeneities in glycogen contents during the second fermentations, FC glycogen distributions' RCVs and histograms' shapes were compared. Figure 5B shows how glycogen RCVs evolved during the fermentation process in all strain/wine cases. In all four cases, changes in RCV values were observed between each and every time point; these changes, globally quite substantial, hint for a variable heterogeneity in yeast populations' glycogen contents throughout the second fermentation (similarly to what was also observed for trehalose). It can also be noticed that for all strains/wine conditions, RCV changes always followed a common trend between each time point, i.e. RCV was detected to increase or decrease simultaneously in each strain/wine condition. This is a rather curious observation, differing from trehalose RCV results. Higher RCVs were observed for both strains in wine 2, particularly from day 19 onwards, yet no analysis was performed to assess their significance. Figure 6B displays selected glycogen histograms corresponding to both strains in wine 1 (results for wine 2 were analogous, with slight dissimilarities). In each of both strains (considered separately), clear differences can be seen between histograms' shapes throughout the second fermentation process, in opposition to what was verified for trehalose (where only minor variations were detected). This emphasizes the already mentioned changing/dynamic heterogeneity. In strain 1, two subpopulations with different glycogen levels seem to have existed during most of the time, eventually "merging" as detected at the last time point (day 85). Strain 2 also appears two have had two subpopulations, most notably prior to inoculation and after the first 24 h of fermentation, which ended up uniting as well. The potential relevance of these results, in the context of a second fermentation, was not assessed and is currently unknown. Further studies must be undertaken to better comprehend these population heterogeneities and their possible implications.

Weak FC signal for glycogen

It is important to refer that acriflavine, with which intracellular glycogen was stained for FC analysis, produced a rather "weak" fluorescence signal, not very much higher than yeast cells'

autofluorescence in the same fluorescence channel (FL1, for green fluorescence; data now shown). Therefore, autofluorescence might have contributed to some of the variation in acriflavine-glycogen fluorescence signals and their frequency distributions (Watson, 1992). Different acriflavine staining protocols (directed at glycogen) are currently published. The one used in this thesis work' is equivalent to the one reported by Brányik *et al.* (2005), but others (Novak *et al*., 2007; Chlup *et al*., 2008) may perhaps provide better results (higher specific fluorescence) and should be tried in the future.

6. Neutral Lipids

Neutral lipids dynamics (FC)

Intracellular neutral lipids were solely assessed by flow cytometry (FC), and average values of FC fluorescence distributions were used for analysing their overall dynamics during the second fermentations (Figure 8).

Throughout the first 48 h after inoculation, strain 1 showed a considerable decrease in neutral lipids content, in both wines. This prompt mobilization of neutral lipids was most likely caused by the need to synthesise new sterols and/or fatty acids, required to repair and restore membrane(s) structure and functionality during and after rehydration in the base wine. This assumption is quite logical, considering that dehydration of industrially propagated wine yeasts (to produce active dry forms) causes substantial cell membrane injuries (Garre *et al*., 2010), which cells must be able to fix. Moreover, inoculation into a base wine exposes yeast cells to high levels of ethanol, which is known to compromise normal membrane structure and integrity, and increase its fluidity; in order to tolerate these disturbing effects, cells may need to modify their membrane composition so as to better stabilize it (Ding *et al*., 2009), a process that requires new building blocks for membrane biogenesis. The low incubation temperature (10ºC) may have also contributed to changes in cell membrane composition (Aguilera *et al*., 2007). Strangely, no evident reduction of neutral lipids levels was detected in strain 2 during the same initial period (in both wines). In this strain, neutral lipids were seen to decrease for the first time only between days 2 and 12, and just in wine 2. These observations are somewhat puzzling. Regardless of the initial disparities between both strains, a

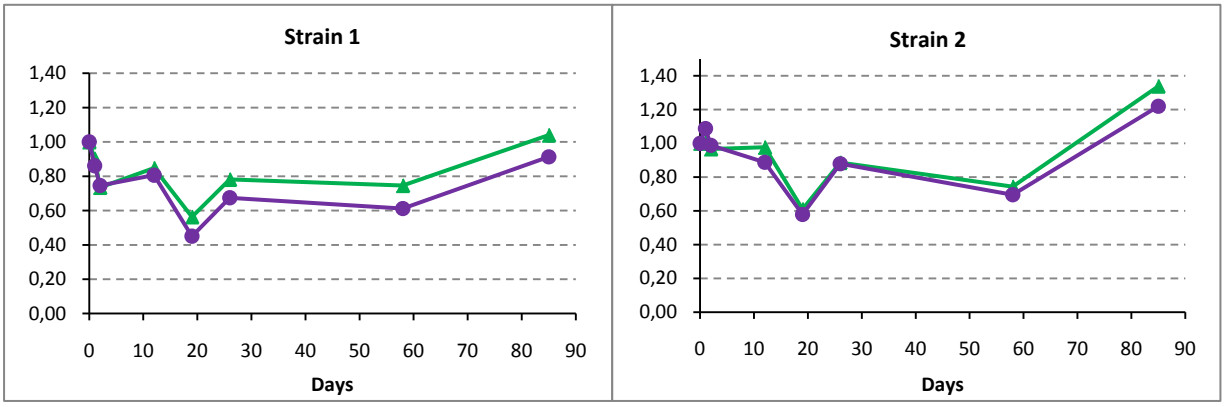

Figure 8. Evolution of yeasts' neutral lipid contents throughout the fermentation process. Presented values are proportions relative to day 0 (before inoculation) of FC neutral lipid levels in base wine 1 (green) and base wine 2 (purple) fermentations. Averages for replicates (3 at time point 0, 2 at the remaining time points) were used. FC analysis was based on averages of fluorescence intensities.

marked decline in neutral lipids content was detected for all strain/wine cases between days 12 and 19. This may reflect a common and delayed adaptation process, associated with extensive new membrane biogenesis and composition changes, in response to sustained (*e.g.* low temperature) and increasing (*e.g.* ethanol and pressure; Fernandes *et al*., 2004) stresses. Between days 19 and 26, neutral lipids reserves were partially replenished, again in all conditions. After a second generalized consumption between days 26 to 58, especially in strain 2, a substantial accumulation took place. This build-up, seen in all conditions, may indicate that no further key changes were introduced in membrane composition, and also that neutral lipids were generally not used as an energy source under glucose and fructose exhaustion, as opposed to trehalose and glycogen.

Globally, each strain showed a comparable neutral lipid production/consumption behaviour in both wines, which indicates that the two wine environments did not influence neutral lipids metabolism very differently. FC average values for neutral lipids were always at least 1.5 times higher in strain 1 than in strain 2 (data not shown); higher neutral lipids content could perhaps be one additional reason for strain 1 higher viability throughout the second fermentation in both wines.

Population heterogeneities in neutral lipids levels

Yeast populations' heterogeneities in neutral lipids contents during the second fermentations were also considered. As before, FC distributions' RCVs and histograms' shapes were compared. Figure 5C illustrates how neutral lipids RCVs changed through the process, for all strain/wine pairs. Like with trehalose and glycogen, the observed RCV variations are highly suggestive of a changing heterogeneity. Moreover, as in the case of glycogen, RCV changes for every strain/wine condition always followed a common trend (between each time point). Figure 5C also clearly shows that RCVs of neutral lipids are globally much higher than those of trehalose or glycogen, meaning a higher degree of overall heterogeneity. Figure 6C exhibits selected histograms, corresponding to both strains in wine 1 (results for wine 2 were fairly comparable). Changes in histograms' shapes throughout the second fermentation are easily seen for both strains, highlighting the dynamic heterogeneity already pointed out. Furthermore, there are obvious differences between strains, *e.g.* prior to inoculation and at day 58. As in the case of trehalose and glycogen, the significance of these particular results and the overall importance of heterogeneity in the process are unknown.

7. Relative gene expression

Endogenous/reference genes

Both *18S* (*RDN18*) and *ACT1* were initially selected to be used as endogenous/reference control genes. Using multiple reference genes for normalization of expression levels is strongly recommended, as it balances possible expression variations of single reference genes and therefore provides more reliable results (Teste *et al*., 2009). It was realized, however, that *ACT1* expression did change considerably throughout the second fermentation in all strain/wine cases (data not shown). For this reason, only *18S* was used for normalization. This was at first unexpected, because *ACT1* is regularly used as a reference gene in expression studies with either laboratory strains (Kaino and Takagi, 2008) or wine strains (Zuzuarregui *et al*., 2005; Garre *et al*.,

2010) of *S. cerevisiae*, being considered to have stable transcription levels under most experimental conditions. This is more often than not just an assumption, because *ACT1* expression stability is not usually assessed in those studies. A systematic validation of potential reference genes such as *ACT1* is nevertheless essential to ensure proper normalization (Ståhlberg *et al*., 2008). Published reports reveal that *S. cerevisiae ACT1* expression may decrease upon entry and during the stationary phase of growth (Choder and Young, 1993; Wenzel *et al*., 1995). As previously stated, the very stressful second fermentation conditions, allied to the physical hindrance effect of the alginate matrix, most probably inhibited yeast growth very early, because no evidence of cell multiplication was found at any time point. Thus, *ACT1* could be expected to have its expression levels diminished very prematurely, and this was indeed observed from day 2 onwards in all four conditions.

Polyadenilated 18S rRNA

In opposition to *ACT1*, *18S* kept quite steady expression levels during the whole study in all four strain/wine conditions (data not shown). For a fixed threshold level in amplification curves, defined at the exponential phase of amplification, *18S* C_T showed a global standard deviation of 0.395 in strain 1 and 0.385 in strain 2 (considering results for both wines). These values may be viewed as reflecting a low dispersion (it was by far the lowest in the study) and therefore a low variation in expression, not compromising normalization to a considerable extent. It should be emphasized that reverse transcription for the production of cDNA was performed using oligo-$d(T)_{20}$ primer; hence, only poly(A)-tailed RNA is reverse transcribed in the process. rRNA is typically assumed to lack a poly(A) tail (Applied Biosystems, 2004). However, preliminary studies performed before the work described in this thesis showed that *S.cerevisiae* 18S rRNA could effectively be reverse transcribed with oligo-$d(T)_{20}$. These results are supported by at least one study, which shows that a fraction of a diverse set of *S. cerevisiae* rRNAs, including 18S, is actually polyadenilated (Kuai *et al*., 2004).

Evolution of gene expression

In all strain/wine conditions, expression levels of all target genes except *ACT1* (which ended up being considered a target gene as well) were seen to considerably decline to various extents between the time before inoculation (day 0) and 24 h (day 1) post-inoculation (Figure 9). This was not anticipated, especially for *HSP12*, whose expression was expected to rise in response to stresses such as ethanol and low temperature. It is known, however, that dry forms of wine yeasts are subjected to different stresses during their industrial production: biomass propagation, for example, is associated with both osmotic and oxidative stresses, which induce *HSP12*, *TRX2* and *GPD1* expression (Pérez-Torrado *et al*., 2005; Gómez-Pastor *et al*., 2010), and oxidative stress also occurs during dehydration (Garre *et al*., 2010). Moreover, in the specific case of encapsulated yeasts, which may be successfully directly inoculated in a base wine, they are most probably prone to withstand ethanol and other harsh conditions at the end of the production process. Therefore, the yeasts used in this study possibly already had elevated mRNA levels of numerous stress responsive genes before inoculation, including the four (*HSP12, TRX2, GSH1* and *GPD1*) that were analysed. Plus, stress protectants coded in those same genes (*e.g.* HSPs) could be expected to be present in high contents in cells. The lower transcript levels seen at 24 h may thus indicate that

yeasts were already well-adapted to tolerate base wines' environments (but see below). Still, this does not seem to agree with the raise in trehalose levels that was observed at 24 h in all conditions.

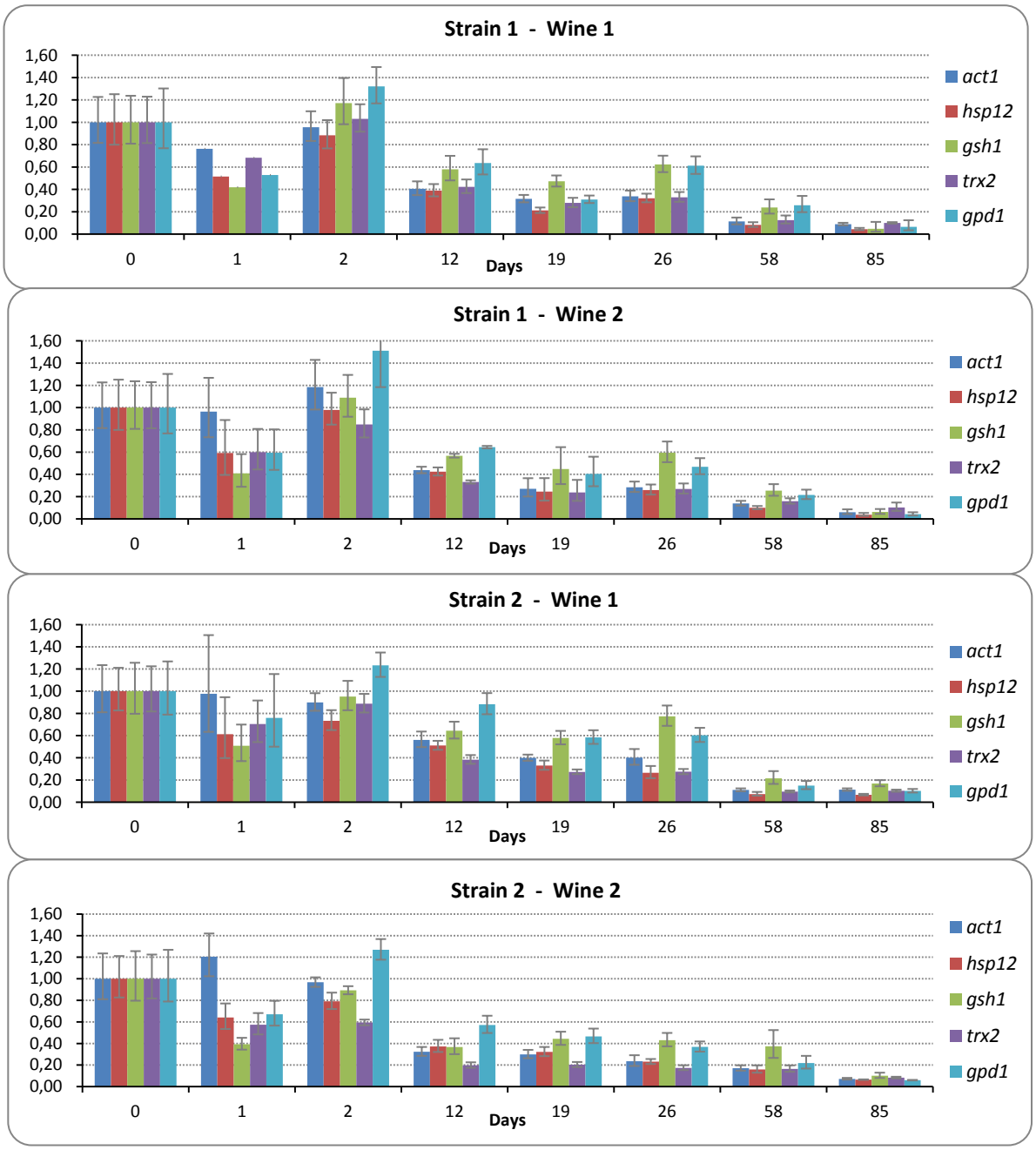

Figure 9. Relative gene expression levels of each target gene throughout the fermentation process. Normalization was performed with *18S* (endogenous/reference control).

Between 24 h and 48 h after inoculation, an increase in relative expression levels of all four stress responsive genes was observed for all strain/wine cases (Figure 9). Globally, this increase was more substantial for strain 1 (in both wines), with expression levels of most genes reaching or even exceeding those determined at day 0; *HSP12* relative expression, for example, was in fact observed to significantly depend on the strain at day 2 (p=0.03). *GSH1*, and most especially *GPD1*, had the highest increase in all strain/wine conditions. Also, *GSH1* and particularly *TRX2* achieved higher levels in wine 1 than in wine 2 (for both strains). These results strongly suggest that base

wines' environments were indeed hostile for yeasts. Even if well-adapted at the beginning of fermentation, yeasts' continuous exposure to base wines' stresses for more than 24 h probably caused progressive cell injuring, ending up triggering the observed response at day 2. Strain 1 reacted more strongly (proportionally to day 0), which may have contributed to better stress tolerance and maintenance of higher viabilities as compared to strain 2. High expression of *GPD1* was probably a response to hyperosmotic stress, caused by sustained exposure to ethanol (Alexandre *et al*., 2001; Walker and Dijck, 2006). *GPD1* is typically induced at the beginning of must fermentations, but in response to high sugar concentrations (Zuzuarregui *et al*., 2005), which are not present in base wines. The increase seen in *GSH1* and *TRX2* was possibly caused by oxidative stress, associated with accumulating ROS due to the presence of dissolved oxygen. Their expression, particularly of *TRX2*, was higher in wine 1, demonstrating that each wine posed different levels of one of more stresses.

From days 2 to 12, a new decline in mRNA levels of all four stress responsive genes was seen in all conditions. Transcriptional stress responses are known to be only transient for many genes (Gasch *et al*., 2000; Causton *et al*., 2001); a common example in winemaking is the quick hyperosmotic stress response at the beginning of must fermentations, in which *GPD1* expression quickly raises and diminishes soon after (Zuzuarregui and del Olmo, 2004). It is thus reasonable to think that the strong transcriptional response observed at day 2 may indeed have been only transient. The more limited transcription of all four stress genes observed at day 12 may nevertheless still have allowed for their products to be kept at sufficiently high and steady levels, required to continuously tolerate existing stresses. Between days 12 and 26, all target genes' expression levels changed to a variable degree, in most cases not very extensively. This occurred for all strain/wine cases. During this period, *GSH1* and *GPD1* maintained the highest relative expression levels among all target genes, suggesting the relevance of cell control over oxidative and hyperosmotic stresses throughout fermentation. From day 26 to 58, expression levels were seen to diminish to a substantial degree overall, a change not verified to the same extent only for strain 2 in wine 2. The lowest (and quite low indeed) expression levels were however only detected at the last time point, day 85. A few different reasons, not mutually exclusive, may be thought of as possibly justifying these last results: *1)* extended exposure to multiple stresses (ethanol, low temperature, pressure, etc.) may have widely affected yeast cell structures and proper cell functioning, compromising elementary processes such as gene transcription; *2)* sugar limitation/depletion, verified since day 58 and expected to change cell metabolism and gene expression, may have contributed to a decrease in expression of the selected target genes; *3)* mRNA stability may have somehow diminished, perhaps due to a higher turnover rate and/or changing physicochemical conditions in the nucleus and/or cytosol; *4)* a substantial decrease in the mRNA to total RNA ratio may have occurred. Regarding this last explanation, one should recall that only total RNA, and not mRNA, was quantified for use in reverse transcription.

8. Principal component analysis (PCA)

With the purpose of better comprehending the overall results of this study, a principal component analysis (PCA) was ultimately performed on the obtained data. PCA is a multivariate data analysis method which provides a visually interpretable overview of the main information of multidimensional and often complex data sets. By plotting principal components, sample groupings may be detected and analysed, and relationships between variables may also be assessed (Rossouw *et al*., 2009).

A total of 32 samples were used for PCA, each representing all replicates of a single strain in a single wine (for time points other than 0) at a single time point by means of averaged values for each variable. Regarding variables, all which were evaluated during this study, a total of 15 (see Figure 10), were considered.

The first two principal components, PC1 and PC2, were found to explain 70.26% and 12.83% (cumulating 83.1%) of the observed variance in the sample data set, respectively; their combined explicative power, being high (≥75%), justified their selection for PCA space representation and sample analysis. Figure 10, a loading plot depicting each original variable loading value for PC1 and PC2, clearly demonstrates that PC1 is substantially characterised by most variables (except for AQ trehalose (F) and FC neutral lipids (J), all others have PC1 loadings superior to 0.7 in absolute value), while PC2 is mainly associated with AQ trehalose (F, -0.81) and wine glucose (B, 0.63). The loading plot also illustrates existent correlations between variables, *e.g.* between MB and FC viabilities, demonstrated earlier, and also between all target genes expression[12] (data not shown).

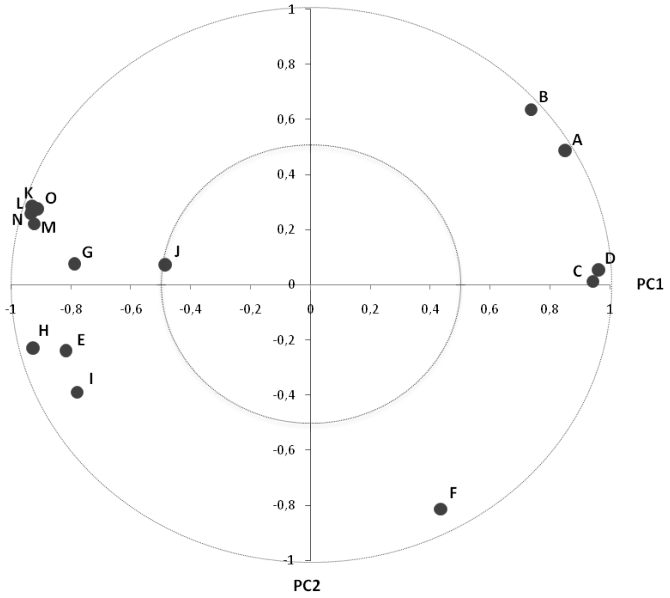

Figure 10. Loading plot of original variables for principal components 1 and 2.

A – Glucose/fructose in wine
B – Glucose in wine
C – MB viability
D – FC viability
E – Total proteins
F – AQ trehalose
G – FC trehalose
H – AQ glycogen
I – FC glycogen
J – FC neutral lipids
K – *ACT1* expression
L – *HSP12* expression
M – *GSH1* expression
N – *TRX2* expression
O – *GPD1* expression

The PCA plane, as defined by PC1 and PC2, is represented in Figure 11, with each sample projected according to its principal component scores (i.e. coordinates on the principal components space). Globally, it is quite evident that sample variation and separation are very much dependent on time. In fact, samples follow a fairly well defined path on the PCA space according to the progression of the second fermentation. Samples from time points 0, 1 and 2 are rather proximate, at the top of the presented PC1 and PC2 scales, and an easily recognizable group can be defined

[12] This would be expected, as all them had a similar evolution on expression levels throughout the study.

with them. From time point 2 onwards, a substantial and progressive separation takes place along both principal components, mainly explained by: changing AQ trehalose levels, reflected through the length of PC2 (higher AQ trehalose translates into more negative PC2 scores); decreasing viability and wine fermentable sugars (glucose and fructose), evidenced by increasingly lower PC1 scores. These PCA results demonstrate the lower discriminating power of the remaining variables in this study. Looking at parts 1 and 2 of Figure 11, it is possible to see that each strain does not present very different PC1/PC2 scores for both wines at each time point, at least until day 19 (strain 1) or 58 (strain 2). Overall, strain 2 seems to have had a more similar behavior in both wines than strain 1.

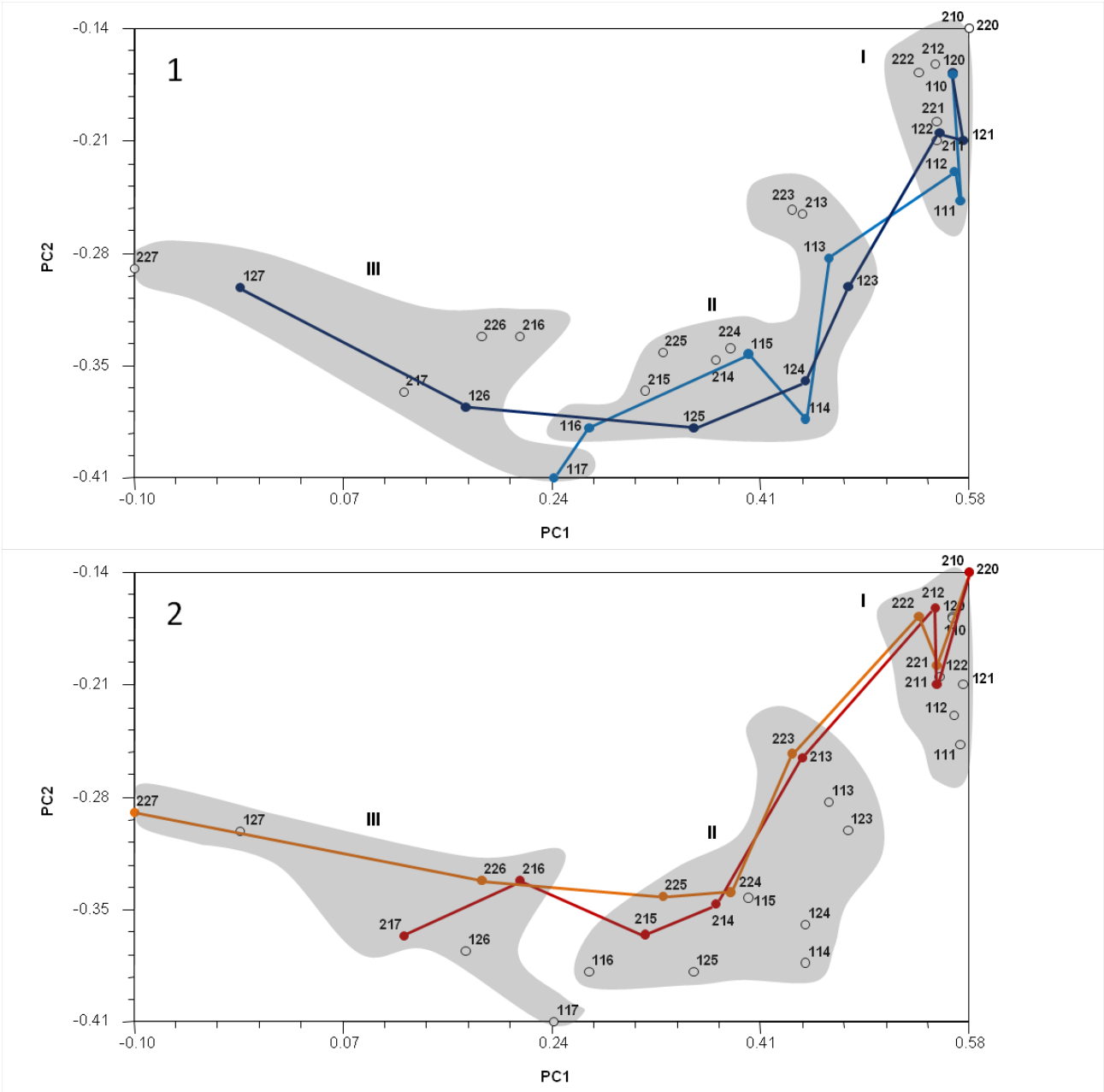

Figure 11. PCA scatter plots, defined by principal components 1 and 2 (representing 83.1% of variance). Evolution of strain 1 and strain 2 samples along fermentation is highlighted in part 1 and part 2, respectively. Shaded areas refer to clusters I, II and III defined on the dendrogram of Figure 12. A three-digit code was used to identify samples: strain (1 or 2) – wine (1 or 2) – time point (0: before inoculation; 1 to 7: 1, 2, 12, 19, 26, 58 and 85 days of fermentation).

PCA samples were also clustered using the Euclidean distance coefficient, which was applied on their principal component scores. Figure 12 represents the resulting dendrogram. Although some

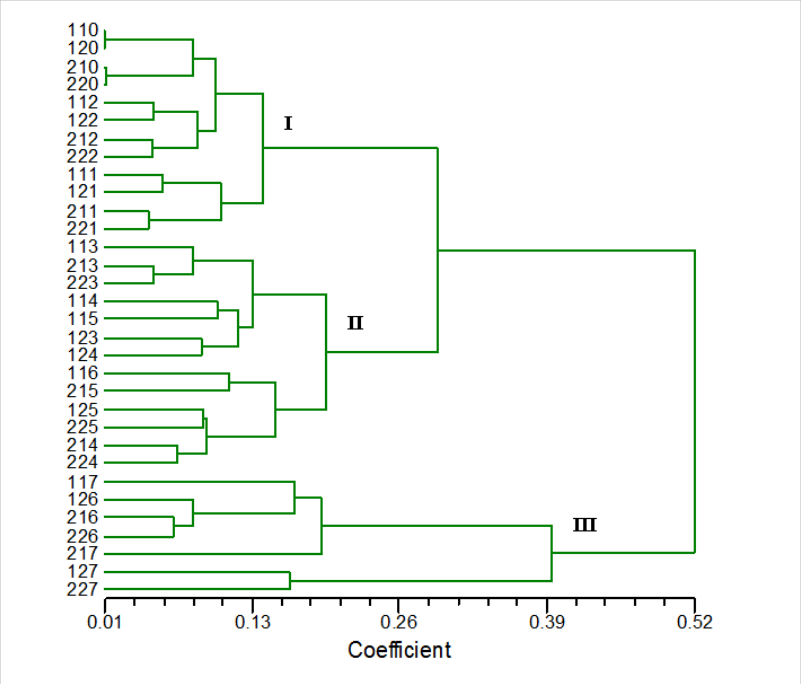

Figure 12. Dendrogram generated from PCA samples using Euclidean distance as association measure and UPGMA as agglomerative algorithm.

clusters very well reflect proximity relationships identified on the PC1/PC2 PCA plot (*e.g.* samples 213 and 223), cases exist for which distances are determined differently (*e.g.* samples 113 and 123), which reveals some information loss in the analysed PCA 2-factor plot. Major clusters of this dendrogram can nevertheless be easily recognized on the PCA plane, most notably the ones comprising: *a)* all samples from time points 0, 1 and 2 (cluster I); *b)* all samples from time points 3, 4 and 5 (cluster II; including also sample 116); *c)* all samples from time points 6 and 7 (cluster III). This again demonstrates that time, associated with fermentation stages, is the key factor determining sample variation and separation. The considered dendrogram was further compared to another one generated from standardized sample values before PCA (using the Pearson correlation coefficient) and both were seen to provide equivalent results.

CONCLUSIONS AND FUTURE PERSPECTIVES

Both strains used in this thesis' work showed very similar fermentative performances in each of both base wines. Thus, it is rather difficult to point out specific cell properties and/or adaptation responses (among the ones analysed) which might be especially relevant in favouring or hindering a second fermentation at very low temperatures. In order to better clarify this issue, further studies (analogous to the one herein described) should be performed with additional strains, known or expected to exhibit higher and/or lower fermentative performances at the tested conditions.

During this thesis' study, assessments of total proteins, trehalose, glycogen and neutral lipids provided important clues on yeasts' vitality and/or adaptation state, being quite informative overall. Still, additional cell biomolecules should be considered in future studies of this kind, for extra and equally useful information to be obtained. For example, fatty acids and ergosterol would be important to analyse so as to determine cell membranes' composition and functional condition, and glutathione levels (GSH/GSSG) would give a hint on cell redox homeostasis.

Despite the lack of or weak correlation between AQ and FC determinations for trehalose and glycogen observed in this work, FC proved to be quite helpful and practical, and its full potential should be further explored in future studies. Cell cycle analysis at the population level (Côrte-Real *et al.* 2002), for example, could be easily undertaken with FC and would provide key additional information on yeasts' population state. The relevance of certain population heterogeneities such as for trehalose or glycogen contents may perhaps be harder to determine, and this is an issue to be further addressed.

Regarding gene expression analysis, it certainly offered some indications on transcriptional stress responses. However, due to the rather transient nature some of these responses may display, they may have been largely overlooked. Selecting additional stress responsive genes for future studies will allow a more comprehensive analysis of this kind. Genome-wide transcription analysis would nevertheless provide the ultimate answer.

To my knowledge, this thesis is the very first report of an extensive second fermentation monitorization especially directed at yeasts' physiology and adaptation state, representing a rather innovative approach in the field of sparkling wine production. In fact, only yeasts' viability and fermentation kinetics determinations are usually performed during a second fermentation process. To this date, similarly broad studies had only been carried out for must fermentations, which still receive privileged attention from researchers in oenology.

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